Novel instrument for automated pK_a determination by Internal Standard Capillary Electrophoresis

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ABSTRACT: The Internal Standard Capillary Electrophoresis method (IS-CE) has been implemented in a novel sequential injection – capillary electrophoresis instrument for the high-throughput determination of acidity constants (pK_a) regardless of aqueous solubility, number of pK_as or structure. This instrument comprises a buffer creation system that automatically mixes within a few seconds four reagents for in-situ creation of the separation electrolyte with a pH range of 2-13, ionic strength of 10-100 mM and organic solvent content from 0-40%. Combined with 1.2 kV/cm and a short effective length (15 cm to the UV detector) fast 20 s electrophoretic separations can be obtained. The low standard deviation of the replicates and the low variation compared to reference values show that this system can accurately determine acidity constants of drugs by IS-CE. A single pK_a can be determined in two minutes and a set of 20 measurements in half an hour, allowing rapid, simple and flexible determination of pK_a values of pharmaceutical targets.

New technologies and strategies for drug discovery and development have evolved considerably over the last few decades adding new opportunities for gathering and integrating information to increase drug discovery success and efficiency[1,2]. Consequently, pharmaceutical companies synthesize a great number of potential drugs and chemical precursors in a relatively short time. To select those which are the most suitable for further development, there is a need for high throughput screening of potential drug candidates as soon as they are available.[3,4]

ADMET (Absorption, Distribution, Metabolism, Excretion and Toxicity) and DMPK (Drug Metabolism and Pharmacokinetic) studies frequently use physicochemical parameters for the understanding of drug properties and processes. One of the main properties that affects the pharmaceutical potential of a compound is the dissociation constant, K_a, (or pK_a in logarithmic scale) since it determines the ionization degree of the compound. In fact, the neutral and ionic forms can have very different physicochemical and biological properties and the pK_a is sometimes decisive for a given application.[4–7]

The determination of acidity constants can be performed by titration, but it is more convenient to use automated methods such as capillary electrophoresis (CE) where multiple targets can be screened rapidly.[8–10] One example is the multiplexed capillary electrophoresis system for high throughput screening for pK_a values which was used to measure pK_as of 103 diverse compounds in an inter-laboratory study.[11] pK_a determination using CE involves measuring the electrophoretic mobility of the target in a number of different pH electrolytes to construct something akin to a titration curve from which the pK_a can be determined. Recently, a new CE approach that uses internal standards (IS-CE) has been developed[12] and applied for a range of different compounds[13] with only three-minutes required per target. The key to this approach is the use of an IS, a compound with a precisely known pK_a similar to that of the analyte (AN). If they are injected together, the differences in the mobilities of the compounds can be directly related to the difference in their acidity. This means that only two electropherograms are needed to determine the acidity constant. Unlike other methods, IS-CE does not need an accurate measure of the electrolyte pH and may correct for interactions of AN with the buffers and possible experimental and systematic errors – such as temperature or pH buffer variations due to electrolysis during a sequence. This method has been evaluated for pK_a determinations of sparingly soluble compounds reaching solubility limits of 10^{-6} mol·L^{-1}.[14] Furthermore, for those drugs with lower solubility, the use of co-solvents and extrapolation procedures have been performed.[15] Consequently, IS-CE is now a reliable, precise and accurate method applicable to any kind of compound

regardless its solubility, number of pK_as or structure. However, commercial CE instruments are big and expensive, and the need to cover all the pH range (from 2 to 13 approximately) requires preparation of a lot of different buffers, and electrolyte preparation is tedious and long, particularly for methanol-water buffers. There are also storage issues when multiple electrolytes need to be kept for prolonged periods of time. To overcome these issues, special instrumentation has been designed and adapted to the IS-CE method to simplify and streamline the process for pK_a determination by using a sequential injection – capillary electrophoresis platform.

Sequential injection – capillary electrophoresis uses a flow-based sample and electrolyte system to introduce solutions into the capillary, and is suited for continuous monitoring applications or the integration of sample handling processes.[16,17] In the present work, a recently developed sequential injection – capillary electrophoresis system[18–21] has been adapted for the determination of acidity constants. The detector was changed to a UV absorbance detector and an automated on-line buffer mixing system developed in order to prepare fresh buffer at any desired pH and co-solvent mixture. The ability to accurately determine a pK_a value in 2 min is shown in aqueous media and mixed aqueous-methanol conditions.

EXPERIMENTAL SECTION

Chemicals and Reagents. Dimethyl sulfoxide (DMSO), methanol (MeOH), potassium chloride (KCl), sodium formate, 2,2-bis(hydroxymethyl)-2,2',2''-nitrilotriethanol (BisTris), tris(hydroxylmethyl)amino-methane (Tris), 2-(cyclohexylamino)ethanesulfonic acid (CHES), 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) of analytical reagent grade were obtained from Sigma-Aldrich (New South Wales, Australia). Standard solutions of 0.5 M hydrochloric acid and 0.5 M sodium hydroxide were from Riedel-deHaën (Seelze, Germany) and anhydrous sodium acetate from AJAX (Sydney-Melbourne, Australia). Solutions were prepared in water from a Milli-Q water plus system from Millipore (Bedford, MA, USA), with a resistivity of 18.2 MΩcm. All studied drugs (internal standard set[12], mefenamic acid and terfenadine) were reagent grade or purer, and were purchased from Sigma-Aldrich, Merck (Darmstadt, Germany) or Carlo Erba (Milan, Italy).

Stock buffer solutions (sodium formate, sodium acetate, BisTrisHCl, TrisHCl, NaCHES and NaCAPS) and pH modifiers (NaOH and HCl) were prepared in aqueous media at 0.25 $mol \cdot L^{-1}$. A 0.05 M of KCl aqueous solution was prepared for the outlet vial.

Stock solutions of ANs and ISs were prepared at a concentration of 1000 $mg \cdot L^{-1}$ and 4% DMSO was added as electroosmotic flow (EOF) marker. They were diluted in water or in a methanol/water mixture (when they were not soluble in water itself). Afterwards, a 1/10 dilution of the stock solution in water was prepared for injection (100 $mg \cdot L^{-1}$, 0.4% DMSO).

All stock solutions were filtered through a nylon mesh 0.45 µm porous size (Agilent Technologies, Santa Clara, CA, USA).

Instrument Design. A schematic diagram of the home-built instrument is shown in Figure 1, based upon a modified design for the rapid separation of inorganic explosive anions recently developed.[18] This IS-CE instrument is composed of five Milligat pumps (MG-5, Global-FIA, Fox Island, WA, USA). The first four were used to create and deliver the desired background electrolyte (BGE) and solvent, and the fifth to pump both AN and IS. A buffer selection valve (7-port selector valve; MXP-7970, Rheodyne, Oak Harbor, WA, USA) was connected to pump 1 to select the appropriate stock buffer solution. A pH modifier valve (2-position; MXP-7980, Rheodyne, Oak Harbor, WA, USA) was connected to pump 2 to select acid or base solution to titrate the BGE to the appropriate pH. Pumps 3 and 4 were connected to water and co-solvent containers, respectively, with the water used to ensure constant ionic strength across all pH values, and the co-solvent used to increase the solubility of compounds not soluble in water. Analyte and internal standard trays were connected to pump 5 by a PEEK (poly(ether ether ketone)) Y-shape piece (P-512, Upchurch Scientific, Oak harbor, WA, USA). A 2-position 4-port injection valve (360T041SHH, NResearch, West Caldwell, NJ, USA) was used to direct sample or BGE to the analytical system. A PEEK tee-shape connector (P-727, Upchurch Scientific, Oak harbor, WA, USA) was used to interface the flow system and the CE capillary. The capillary inlet was fixed at a constant position close to the centre of the interface with the help of a piece of capillary (360 µm o.d.) introduced through the horizontal part of the tee-piece. The outlet side of the capillary was immersed in a 5 mL vial filled with 50 mM KCl. A stainless steel syringe needle was cut to yield a 2 cm long, 0.51 mm i.d. tube and was employed as outlet and ground electrode and connected to the interface through the waste tubing. An isolation valve (HP225K021, NResearch, West Caldwell, NJ, USA) was linked on the waste tubing at the T piece outlet to control solution direction either to capillary or to waste.

The four channels used to make the BGE were connected into a microfluidic 3D micromixer, based upon Baker's transformations[22], printed using a Miicraft 3D printer (Hsinchu, Taiwan). The object was designed in CAD software and converted into STL with triangle facets. The digital 3D model was sliced into 2D cross section layers of 50 µm depth and printed using colourless cream resin (epoxy-acrylate, resistant to acid/base solutions and different solvents like methanol, decanol, and dichloromethane[23]). The 3D micromixer was designed with four channels (from pump 1 to pump 4) and connected by a "Y" shape as shown in Figure 1, creating laminar flow in four different sections. Subsequently, this 4 laminar-flow was mixed by splitting and recombining the flows at a perpendicular angle in a mixing unit. A previous study[23] found that 0.1 µg/mL fluorescein and 0.1µg/mL rhodamine were mixed completely after passing through 4 mixer units. As there were acid-base reactions and solvent-water mixtures, a total of 6 units in a row were printed to ensure a complete

mixing. The entire chip was 32 mm × 20 mm × 14 mm (width × depth × height), yielding 0.5 mm wide and deep squared micro-channels for the solution and 1.6 mm cylindrical channels to squeeze in the PEEK tubing. Two miliGAT pump pressure release valves of 100 psi were positioned after the 3D micromixer and after the sample pump.

Separation was driven by an EMCO 4300N high-voltage power-supply (Sutter Creek, CA, USA) working with normal polarity with the cathode (-) electrode immersed in the outlet vial. A commercial ActiPix D100 imaging detector was purchased from Paraytec (York, UK). The detector uses a 9 mm × 7 mm active pixel sensor array made up 1280 × 1024 individual 7 µm pixels. The xenon light source was filtered using a 214 nm interference filter. The sample rate was 10 Hz. The detector head was positioned 10 cm from the outlet of the CE capillary. The detector was insulated from the high voltage supply using a 3D printed case. Data acquisition was obtained by Paraytec ActiPix D100 Online Mode software.

The system was controlled from a laptop using USB to RS-485 converter (GlobalFIA, Fox Island, WA, USA) serial connections for the Milligat pumps. The injection valve, isolation valve and high voltage power supply were interfaced to the computer using NI DAQ system (USB-6212). BGE and pH modifier valves were also interfaced but using NI DAQ system (USB-6008). Total system control, except data acquisition was achieved using software LabView v11.0. Both interfaces and software were from National Instruments (Austin, TX, USA). The same software was used to monitor both voltage and current provided by the power supply. Figure 2 shows a photograph of the prototype instrument with its main parts.

The entire system was operated in a small cabinet with a Ranco ETC-211100-000 Digital Temperature Controller (Pain City, OH, USA) connected to a cooler and a heater. Temperature was set to 25±1 ºC in whole cabinet using this controller and a fan.

System operation. Operational sequence steps of the IS-CE system are described in Table 1. Briefly, the tee-interface was filled with the BGE and the capillary rinsed by closing the isolation valve for 20 s. Subsequently, a mixture of IS and AN were injected by firstly filling the interface and secondly closing the isolation valve for 5 s. After injection, the tee-piece was cleaned using the BGE. Finally, the BGE flow rate was changed (0.02 µL·s^{-1} for weak bases, 0.3 µL·s^{-1} for weak acids), isolation valve closed and HV applied to facilitate a pressure-assisted electrophoretic separation. These conditions were selected in order to minimize analysis time and consumption of reagents.

To prepare the aqueous buffers at the desired pH and constant ionic strength of 50 mM, calculated flows of 0.25 M HCl or 0.25 M NaOH were added to a constant flow of the 0.25 M stock buffer solution (always 20 % of the total BGE flow rate). Buffer stock solution was chosen according to the buffering pH range of each buffer (pK_a ± 1). Finally, water was added to dilute the buffer solution until a constant ionic strength of 50 mM. For MeOH-H_2O mixtures, calculations were performed for each specific mixture keeping the same percentage of stock buffer solution. Buffer pK_a values and pH scale shifts due to methanol (when measuring in methanol-water solvents) were taken into consideration. For extremely basic pH (around 13), the appropriate flow rate of 0.25 M NaOH was directly mixed with the desired proportion of MeOH/H_2O maintaining the ionic strength constant. In this manner, all the useful pH range (from 2 to 13) at any percentage of methanol mixture between 0.0 and 40.0 % (v/v) can be covered.

Electrophoretic conditions. Separations were performed using fused-silica capillaries (25 µm I.D., 360 µm O.D. and 25 cm in length, 15 cm to the detector) obtained from Polymicro Technologies (Phoenix, AR, USA). Slightly low I.D. capillaries allow working at higher electric field strength without losing much sensibility. Sample and internal standard were injected hydrodynamically by closing the isolation valve for 5 s at 0.5 µL·s^{-1}. Separation was performed at 1.2 kV/cmand normal polarity under pressure by closing the isolation valve while BGE was flushing at 0.02 µL·s^{-1} (bases) or 0.3 µL·s^{-1} (acids). Capillaries were conditioned for the first time by flushing 1 M NaOH for 2.0 min at 1 µL·s^{-1} and then 2.0 min with water at same rate. For routine analysis, the capillary was rinsed for 2.0 min with 0.25 M NaOH and 0.5 min with water at the beginning and ending of the session using pump 2 and 3 of the system in Figure 1. When the instrument was not in use, stock solutions were removed and stored in a refrigerator at 4ºC and all tubing and pumps were flushed for 2 min with a solution of 15% MeOH/water (v/v).

pK_a determination by IS-CE method. The procedure for acidity constant determination by IS-CE has been previously reported.[12] Briefly, the method is based on the use of an IS with pK_a similar to that of test compound (ΔpK_a < 1), choosing as first approximation the pK_a predicted for the test compound using ACD/Labs software.[24] Then, mobilities of the IS and the test compound are measured in two different buffers: a buffer in which the AN and the IS are completely ionized (pH ≫ pK_a for an acid or pH ≪ pK_a for a base); and a second buffer in which both are partially ionized (pH in the range pK_a ± 1). From these mobility measurements the pK_a of the test compound can be directly obtained if the pK_a of the IS is accurately known.

For determination of aqueous pK_a of insoluble compounds, acidity constants (pK_a) at different ratios of hydro-organic mixtures are determined. Then, the pK_a values are extrapolated to 0% organic solvent by the Yasuda-Shedlovsky equation.[25] This mathematical expression performs a lineal extrapolation between pK_a values through the eq 1.

$$pK_a + \log[H_2O] = \frac{a}{\varepsilon} + b \qquad (1)$$

In this equation log[H_2O] is the logarithm of the molar water concentration of the given solvent mixture, and ε is the electric permittivity of the binary solvent.[26] From the plot of the pK_a of a compound in a given methanol/water mixture vs. the inverse of the electric permittivity of the binary solvent a linear relationship should be obtained. Extrapolation to pure water provides the aqueous pK_a of the compound using ε = 78.3 and log[H_2O] = 55.5.

RESULTS AND DISCUSSION

The IS-CE method recently developed[12] has become a powerful and high-throughput tool for pK_a determination in drug discovery and development. However, there are some limitations regarding the previous system. A large number of BGEs are to be prepared *a priori* and stored in the instrument autosampler. This reduces sample capacity decreasing the usefulness of this approach. Moreover, pH instability of aqueous and solvent-water buffers is always a problem. Therefore, it would be ideal to have the electrolyte made on-line according to the requirements for each target.

For the system to be functional, it is necessary to be able to generate on-line buffers with pH ranging from 2-13 approximately. This could be achieved in two main ways. The first is to mix several different buffer constituents (for instance, NaHCOO, pH 2.6-4.8; BisTrisHCl, pH 5.5-7.5; TrisHCl, pH 7.0-9.0; NaCAPS pH 9.4-11.6) to obtain the desired pH. However, this approach has some disadvantages: the pH and ionic strength are difficult to control as they all are weak acids and bases and ion-pairs between electrolytes have not been studied. In the second and better approach, stock buffer is selected using a valve and the pH is adjusted with strong acid or base, which is instrumentally simpler as it requires 2 fewer pumps. Another 2 pumps can be used to add water to dilute the buffer and ensure constant ionic strength and organic solvent to allow pK_a determinations of water-insoluble compounds. This approach also has the added advantage that acid, base and water are available on-line for conditioning capillaries if necessary.

BGE considerations. Several different buffers have to be used in order to cover the entire pH range from 2-13. These buffers must be adequate as running buffers in CE and the ionic strength has to be kept constant throughout the series.[27,28] Therefore, it is important to select the most appropriate common CE BGEs. In addition, there can be specific interactions with the ANs and the BGE components. In a previous study[29] we evaluated many buffer solutions used in the determination of acidity constants by CZE observing interactions between some buffers and the ANs which led to inaccurate pK_a determinations. As a result of this work, a set of buffers which showed no deviations due to specific interactions were identified. From this, the following 6 BGE components were identified for use in the IS-CE instrument developed here: $HCOOH/HCOO^-$ (pK_a 3.75), CH_3COOH/CH_3COO^- (pK_a 4.76), $BisTrisH^+$/BisTris (pK_a 6.46), $TrisH^+$/Tris (pK_a 8.08), CHES/$CHES^-$ (pK_a 9.55) and CAPS/$CAPS^-$ (pK_a 10.40).

One of the advantages of the IS-CE method is that accurate external measure of the buffer pH is not needed. Small pH variations due to fast capillary conditioning, electrolysis and CO_2 absorption from the atmosphere do not affect to the pK_a determination since the pH is always measured inside the capillary by the internal standard. What cannot be corrected for is changes in ionic strength and this must be the same for the two buffer solutions in which the mobilities are measured. For this reason, buffers must be prepared at the desired pH at constant ionic strength, the latter with a high degree of accuracy. Therefore, the accuracy and precision of the buffer pumps is of vital importance in order to obtain appropriates mixtures. Milligat pumps were selected on their operational and technical specifications: speed between 0.0005-167 $\mu L \cdot s^{-1}$ giving a volume precision of < 0.08 % dispensing 1250 µL and < 0.3 % for 125 µL.

Accuracy of on-line aqueous BGE mixtures. The reliability of buffer pH was evaluated by programming 30 different pH values (from 2.8 to 12.7) with 4-6 pH values from each BGE stock into the on-line buffer generation system. The effluent was collected after the 3D micromixer and the pH measured using a conventional pH-meter. The measured pH ($pH_{(m)}$) values were compared to the calculated ones ($pH_{(calc)}$) as shown in Figure 3. Fitting parameters of the overall data is presented in the following equation:

$$pH_{(m)} = (1.000 \pm 0.004)\ pH_{(calc)} + (-0.01 \pm 0.03) \quad (2)$$

$N = 30$, $SD = 0.08$, $F = 73377$, $R^2 = 0.9996$

The slope and intercept of the correlation are not significantly different from 1 and 0, respectively, for a 95% confidence level. Therefore, these results demonstrate the good agreement and also consolidate the desired pH with respect to the measured one for all the pH range used.

Regarding the maintenance of a constant ionic strength, pK_a values of 8 internal standard (acids and bases) from the reference list[12] were determined by the classic CE method using the home-built instrument. In the classic method, the effective mobility (μ_{eff}) of the analyte is measured at pH values within the working range of CE. The plot of the mobility vs. pH gives a sigmoidal curve with an inflection point when $pH = pK_a'$ (acidity constant at working ionic strength). Consequently, this acidity constant is determined by fitting the corresponding parameters through eq 3.

$$\mu_{eff} = \frac{\mu_{HX^z} + (\mu_{X^{z-1}})10^{pH-pK_a'}}{1+10^{pH-pK_a'}} \quad (3)$$

where μ_{HX^z} and $\mu_{X^{z-1}}$ are the limiting electrophoretic mobilities of the subscript species. pK_a' is related to the thermodynamic pK_a by the activity coefficients, calculated by means of the Debye-Hückel equation.[13] Figure 4 shows the experimental mobilities for the selected internal standards. For those determinations, the capillary was previously rinsed for 3 min with the buffer to ensure that it was conditioned at the desired pH. The curve obtained from the fit is plotted as a line in this figure, and the obtained pK_a values calculated by eq 3 are listed in Table 2, where they are compared to those obtained previously[12]. The fitting parameters indicate an acceptable correlation between pH and mobility and the biggest difference between the two methods for pK_a determination was 0.05. From this it can be concluded that the ionic strength remains constant using the on-line buffer generation system.

Determination of acidity constants by IS-CE. Several acidic and basic compounds with reference acidity constant values were selected as test compounds to evaluate the automated IS-CE system. Some of the reference

values were taken from the internal standard list for this method,[12] and some others were previously determined by the IS-CE method.[15] All of these values have been previously established using commercial Beckman (Palo Alto, CA, USA) and Agilent Technologies (Santa Clara, CA, USA) capillary electrophoresis instruments. Compounds listed in Table 3 show the reference acidity constant at zero ionic strength and 25 °C. This table also shows the IS selected in each determination, the number of determinations, the experimental pK_a calculated with its standard deviation, and its difference from the reference value.

Very small differences were obtained between the acidity constants determined by IS-CE using the home-built CE system and commercial CE equipment. All values are within 0.07 from the reference value and precision is excellent with standard deviation of less than 0.05. In some cases such as papaverine, *N*,*N*-dimethyl-*N*-phenylamine, ibuprofen and terfenadine, the pK_a were determined using two different internal standards with good agreement.

BGE methanol-water mixtures. In order to determine acidity constants of compounds sparingly soluble in water, the performance of the instrument when using methanol-water mixtures as solvent in the BGE was tested. It is well-known that small proportions of organic solvent in water change the acidity constant since it affects the relative permittivity and hydrogen-bond donor capacity. Therefore, hydro-organic buffers can be tested by the respective shifts of acidity constant at different proportion of solvent. The pK_a of ibuprofen was determined using benzoic acid as the internal standard at increasing percentages from 2% MeOH/H_2O (v/v) to 40%. At each level of MeOH mobilities were measured at two pH values as for the completely aqueous system. Figure 5 shows the electropherograms from 2% to 40% of MeOH at a pH where ibuprofen and benzoic acid are completely ionized. This figure demonstrates that migration time increases proportionally with methanol ratio. As voltage and ionic strength were constant during each run, these changes can be attributed to the decreasing conductivity, changes in viscosity as well as changes in the pH scale and the acidity constants of buffers due to the increasing percentage of MeOH. Consequently, this causes mobility shift and non-linear pK_a variations of ibuprofen with respect to the % MeOH (Figure 6). In a previous work,[15] we established the reference acidity constants of all the IS set at 10, 20, 30 and 40% MeOH-H_2O, including ibuprofen. In order to demonstrate the accuracy of the experimental pK_a values, Figure 6b also includes the experimental values at 10, 20, 30 and 40% MeOH-H_2O determined by a commercial CE instrument. The overall results and Figures 5 and 6 demonstrate the good mixing of methanol-water buffers as well as the ability to combine in a few seconds different parameters to create a desired pH and organic mixture buffer.

The addition of MeOH allows pK_a values to be determined for mefenamic acid and terfenadine, which are unable to be determined using 100% aqueous buffers because they are insoluble. For these compounds aqueous pK_a values were extrapolated by Yasuda-Shedlovsky approximation. To validate this approach, ibuprofen was also determined using completely aqueous BGE and also MeOH/H_2O mixtures (from 2% to 40%), obtaining average experimental values of 4.48 and 4.45, respectively. In case of mefenamic acid and terfenadine, comparing the acidity constants determined by this prototype and the commercial CE similar values were obtained (Table 3). The low standard deviation of the replicates and the low variation with respect to the reference values shows the system can accurately determine acidity constants of drugs by the IS-CE method.

Throughput of the home-built CE system. Using the home-built automated IS-CE instrument, faster separations were obtained when compared to commercially available instruments from Beckman or Agilent technologies.[12,15,30] This is primarily due to the much higher electric field strength (1.2 kV/cm). With lower internal diameter (25 µm) and good refrigeration, detrimental Joule heating was avoided.[31,32] Closing the control solenoid and varying the BGE pump speed allows different pressures to be generated to provide faster separations, to avoid current fluctuations and to constantly fill the capillary with fresh buffer. All these conditions allow electropherograms to be obtained within 20 to 60 s, with the MeOH/H_2O mixtures being slightly longer due to the increase in viscosity. Figure 5 shows an example for two acidic compounds in different methanol mixtures.

The on-line buffer generator is the biggest advantage of this IS-CE instrument compared to those commercially available, and is the first to feature on-line electrolyte generation. The mixing system avoids instability, environmental and human errors since it prepares a new buffer each time, consuming low quantities of stock solutions. The software only requires a pH and percentage of methanol to be inserted to fill the capillary with fresh solution within a few seconds.

Using the IS-CE method with this CE system increased the throughput for acidity constant determination. For a single compound just two minutes are needed to determine its acidity constant and for a set of 20 just half an hour. 20 pK_a values each at a different MeOH/H_2O mixture were determined for ibuprofen in half an hour with no preparation of the buffers beforehand, making this a simple and quick approach to the determination of pK_a values in a range of experimental conditions.

CONCLUSION

An automated CE system has been designed and constructed for the implementation of high-throughput IS-CE to determine acidity constants of any bioactive compound. The instrument has been built from commercially available parts with a 3D micromixer designed and printed to combine four different reagents into one single homogeneous flow. Good reliability of pH and ionic strength of buffers were obtained from pH 2 to 13 in aqueous media and several methanol-water mixtures (until 40%). With short capillaries of just 15 cm to the detector, high voltages

(30 kV) and applying pressure during the separation, separations of 20 s were obtained, and 2 min required for a pK_a determination. Furthermore, the ability to determine pK_a using MeOH at 20 different concentrations could be performed in 30 min, allowing the calculation of acidity constants of water-insoluble compounds. The low standard deviation of the replicates and the low variation compared to the reference values show this system can determine acidity constants of drugs by the use of the IS-CE method in a fast and reliable way.

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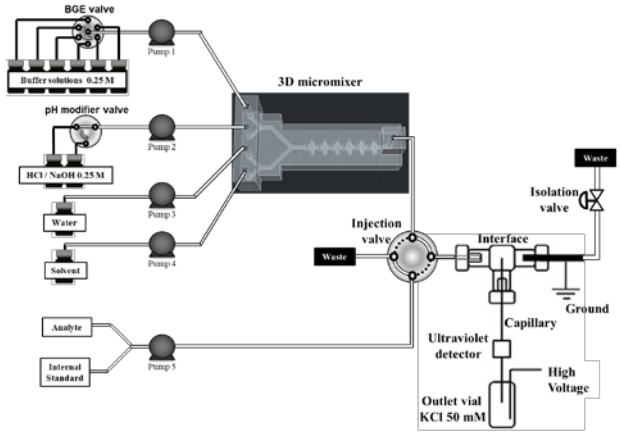


Figure 1. Schematic diagram of the IS-CE system.

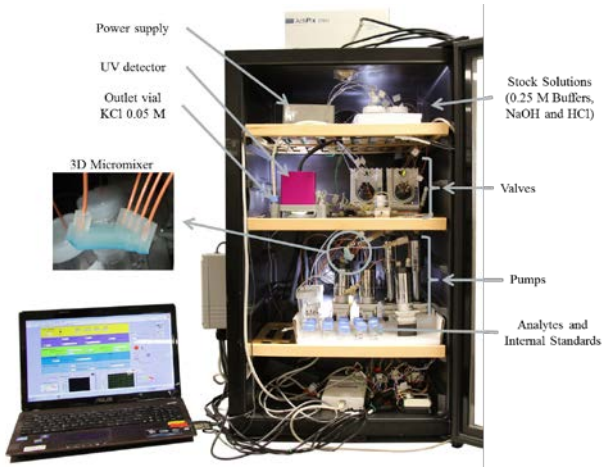


Figure 2. Photograph of the automated IS-CE system.

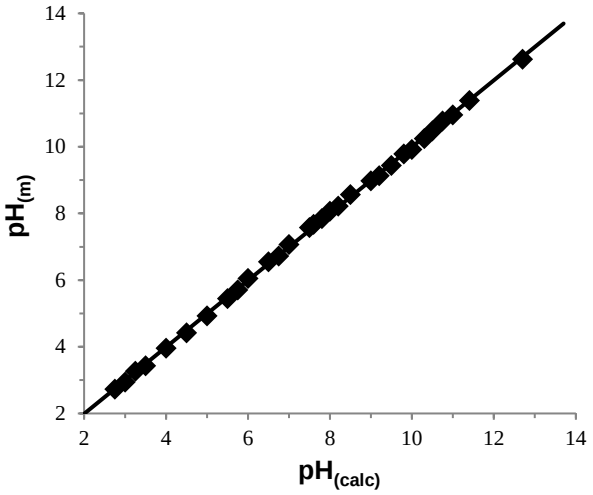


Figure 3. pH of the BGE calculated ($pH_{(calc)}$) as a function of the measured pH after the 3D micromixer using conventional pH-meter ($pH_{(m)}$)

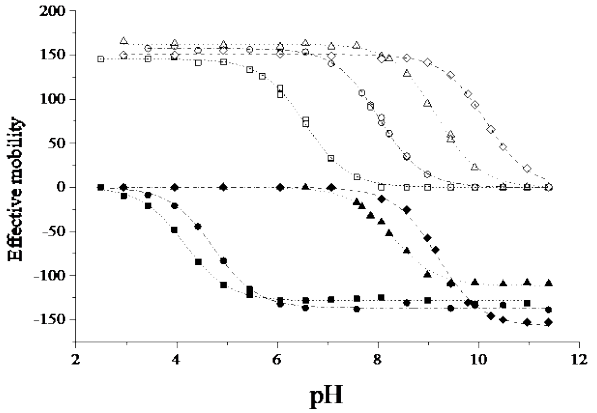


Figure 4. Electrophoretic mobilities ($\times 10^4$ cm^2 V^{-1} min^{-1}) of basic (white) and acidic (black) internal standards vs. pH. Markers are experimental mobilities and fitted lines are obtained by eq 3. (□;------) papaverine; (○;– · –) lidocaine; (Δ;·····) diphenhydramine; (◊;– – –) nortryptyline; (■;------) benzoic acid; (●;– · –) nicotinic acid; (▲;·····) methylparaben; (♦;– – –) 4-bromophenol. Electrophoretic conditions: 50 mM ionic strength, normal polarity at 30 kV, 25 ºC.

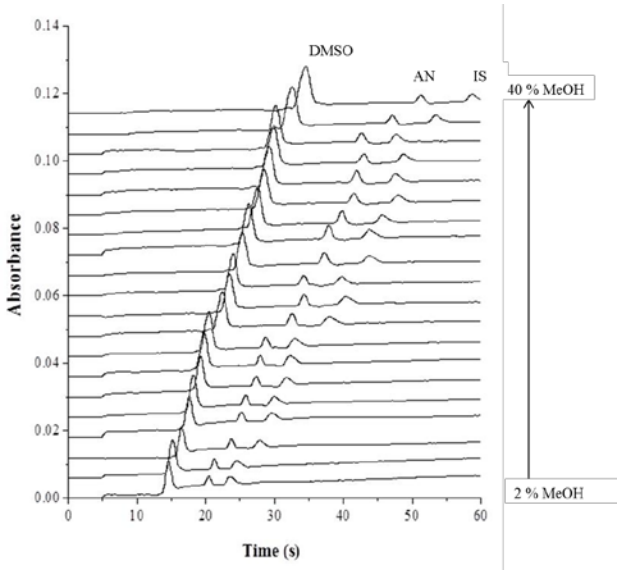


Figure 5. Electropherograms from 2 % to 40 % of MeOH (v/v) of ibuprofen (AN, analyte) and benzoic acid (IS, internal standard) using DMSO as electroosmotic flow marker. Conditions: ionic strength of 0.05 (CHES/CHES$^-$), -30 kV, BGE flow rate 0.3 μL·s^{-1} during separation (isolation valve closed).

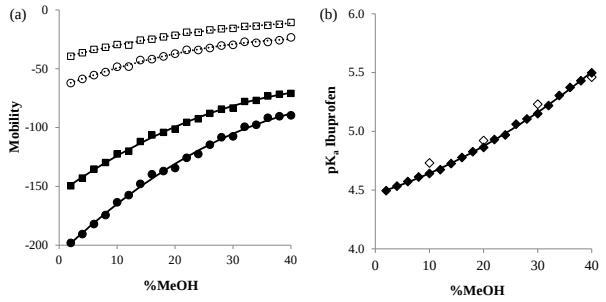


Figure 6. Acidity constants (pK_a) at several MeOH/H_2O mixtures (from 2% to 40%). (a) Mobility ($\times 10^4$ cm^2 V^{-1} min^{-1}) versus % MeOH (v/v) of ibuprofen (□; ■) and benzoic acid (○; ●) at pH

values where they are partially and totally ionized. Fitted solid lines show the mobility at pH when they both are completely ionized, and fitted dashed lines when they are partially ionized. (b) pK_a of ibuprofen at 0 ionic strength and 25 °C as a function of % MeOH (v/v). Solid line is the fit to a quadratic polynomial equation ($R^2 = 0.9992$) and empty symbols (◇) are the reference pK_a of ibuprofen at 10, 20, 30 and 40% of MeOH-H_2O determined by commercial CE equipment.[15]

Table 1. Sequence operation for the IS-CE system.

	Setup operation		Injection valve position	Isolation Valve	BGE Total Flow-rate ($\mu L \cdot s^{-1}$)	Sample Flow-rate ($\mu L \cdot s^{-1}$)	Time (s)	Volume dispensed (μL)
1*	BGE system cleaning		1	Open	30	-	5	150.0
2*	BGE capillary cleaning		1	Close	1	-	20	30.0
3	Sample interface cleaning		2	Open	-	30	10	300.0
4	Equilibrate		2	Open	-	0.5	2	1.0
5	Sample injection		2	Close	-	0.5	5	2.5
6	BGE interface cleaning		1	Open	30	-	5	150.0
7	Separation:	Bases	1	Close	0.02	-	60	1.2
		Acids	1	Close	0.30	-	60	18

* Steps 1 and 2 were operated when buffer pH was changed.

Table 2. Comparison of experimental pK_a values at 25 °C and zero ionic strength obtained with the automated prototype using the classic method, and literature pK_a values obtained by the IS-CE method [12](statistics of the fittings to eq 3 are also shown).

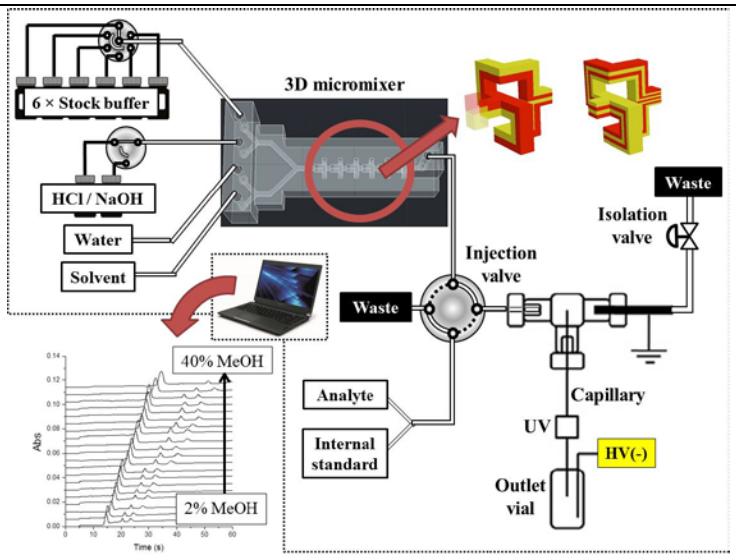
	IS-CE method[12]	Classic method (this work)				
Compound	**pK_a**	**$pK_a \pm s$**	**R^2**	**SD**	**F**	**ΔpK_a***
Base						
Papaverine	6.41± 0.07	6.46 ± 0.01	0.9989	4.39	23151	-0.05
Lidocaine	7.93± 0.01	7.93 ± 0.01	0.9990	4.02	31428	0.00
Diphenhydramine	9.08± 0.02	9.07 ± 0.02	0.9982	8.32	15809	0.01
Nortryptyline	10.08± 0.01	10.05 ± 0.02	0.9976	8.18	13442	0.03
Acid						
Benzoic acid	4.22± 0.03	4.22 ± 0.02	0.9980	4.85	17430	0.00
Nicotinic acid	4.85± 0.03	4.82 ± 0.02	0.9984	5.66	13366	0.03
Methylparaben	8.35± 0.03	8.37 ± 0.03	0.9959	9.15	4380	-0.02
4-Bromophenol	9.28± 0.01	9.27 ± 0.02	0.9973	11.54	6346	0.01

*ΔpK_a: pK_a (IS-CE method) - pK_a (classic method)

Table 3. Thermodynamic acidity constants determined by the Internal Standard Capillary Electrophoresis system ($pK_{a(exp)}$) and comparison with the reference pK_a values ($pK_{a(ref)}$) values established using commercial instruments. $\Delta pK_a = pK_{a(ref)} - pK_{a(exp)}$. N: number of determinations.

Analyte	**$pK_{a(ref)}$**	**IS**	**N**	**$pK_{a(exp)} \pm s$**	ΔpK_a
Aqueous Buffers					
Papaverine	6.41[a]	4-*tert*-butylpyridine	8	6.43 ± 0.04	-0.02
		2,4-Lutidine	3	6.43 ± 0.02	-0.02
Clonidine	8.10[a]	Lidocaine	12	8.07 ± 0.04	0.03
N,*N*-dimethyl-*N*-phenylamine	8.95[a]	Diphenhydramine	12	8.97 ± 0.05	-0.02
		Propranolol	7	9.02 ± 0.05	-0.07
Diphenhydramine	9.08[a]	*N*,*N*-dimethyl-*N*-phenylamine	13	9.06 ± 0.05	0.02
Ibuprofen	4.49[a]	Benzoic acid	5	4.47 ± 0.04	0.02
		Nicotinic acid	5	4.48 ± 0.06	0.01
Phenol	9.89[a]	4-Bromophenol	3	9.92 ± 0.03	-0.03
		Paracetamol	3	9.89 ± 0.02	0.00
MeOH/H_2O Buffers					
Ibuprofen	4.49[a]	Benzoic acid	20	4.45 ± 0.06	0.04
Mefenamic acid	4.17[b]	Benzoic acid	4	4.11 ± 0.03	0.06
Terfenadine	9.19[b]	*N*,*N*-dimethyl-*N*-phenylamine	4	9.26 ± 0.04	-0.07
		Diphenhydramine	4	9.22 ± 0.03	-0.03

[a] Values taken from reference.[12]
[b] Values taken from reference.[15]



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