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Determination of pK_a values of basic new drug substances by CE

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Abstract

During the discovery phase for chemical entities as potential new drugs, the determination of pK_a values can be a very valuable parameter in the selection process. In general, at the very beginning, only small amounts of these active pharmaceutical ingredients (API) are available for analytical characterisation. One drawback of traditional methods such as titration and UV spectroscopy is that they require large amounts of high purity material. As an alternative technique, a method by capillary electrophoresis (CE) which is based on migration times or mobilities of the ionic species over a range of pH values has been evaluated for the determination of pK_a. From the relationship between measured mobilities and pH of electrolyte, a S-shaped curve was obtained and pK_a values determined for some new API entering the screening phase. On the basis of our experience, the pK_a determination by CE of early phase compounds can easily be performed with a very small quantity of material and in addition with insoluble compounds. pK_a values, similar to those earlier observed by potentiometric titration or computational calculations, were obtained. The reported technique using CE can easily be automated and is able to cope with the high throughput needed at the screening stage of lead optimisation.

Keywords: Capillary electrophoresis; pK_a determination; Lead optimisation

1. Introduction

During the lead optimisation phase, where large numbers of molecules are evaluated in parallel, the determination of physicochemical properties is essential to ensure adequate characterisation and quality of development candidates. In addition to parameters such as solubility and lipophilicity, the knowledge of dissociation constants (pK_a's) of these new chemical entities is of fundamental importance in order to provide information for scientists working on:

- absorption, distribution, metabolism, excretion (ADME);
- chemical reactivity, salt formation, or purification processes;
- formulation development;
- chromatographic separations (retention times and selectivity dependence of mobile phase pH).

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Traditional techniques for pK_a determination such as potentiometric titration or spectroscopic methods often require a large quantity of pure material.

However, during screening of large numbers of potential new pharmaceutical entities, only small amounts of impure material are available for analytical characterisation, and it is, therefore, essential that alternative methods should be investigated.

With titration methods or UV spectroscopy, solubility limitations and purity of compounds are potential drawbacks. Furthermore, with spectroscopy (UV/Vis), spectral differences between neutral and ionic species are a pre-requisite.

With both techniques, sample and solution preparation is often tedious and may involve special requirements (e.g. carbonate free solution).

Other approaches such as NMR, conductivity, or calorimetry are restricted to specific applications. Computational predictions are often a good alternative for poorly soluble or impure compounds but may require some knowledge of similar structures in order to be accurate.

Finally, the automation of these techniques is not always straightforward.

HPLC requires only small amounts of material but, HPLC using silica-based columns has a limited pH range, and a polymeric column can be problematic.

Capillary electrophoresis (CE) has evolved considerably over the last decade and is particularly effective in separating ionic species. It has, therefore, been investigated in several occasions [1–4] for the determination of dissociation constants.

The CE method only involves measurement of the ionic mobility (migration time) of the analyte as a function of the pH of the CE mobile phase. As this technique can be easily automated, data can be generated in a timely manner.

The aim of this work is to describe a fast and automated CE technique that requires only milligram quantities to determine the pK_a's of API and which is more selective in separating sample mixtures.

2. Theoretical background

2.1. Definition of pK_a

The dissociation of a substance into ions or ionisation is given by the equation:

$$HA \Leftrightarrow H^+ + A^- \tag{1}$$

With the ionisation coefficient represented by:

$$\alpha = \frac{\text{Dissociated molecules}}{\text{Total number of molecules}} \tag{2}$$

For an acid HA, we can assume that $\gamma_{HA,}$ the activity coefficient of the undissociated acid is equal to 1, the equilibrium or dissociation constant is given by:

$$K_a = \frac{[H^+][A^-]}{[HA]} \gamma_{A^-} \cdot \gamma_{H^+} \tag{3}$$

with γ_{A^-} and γ_{H^-} the activity coefficients.

For a conjugate base A^- the dissociation constant is represented by:

$$K_b = \frac{[OH^-][HA]}{[A^-]} \text{ activity coefficients} \tag{4}$$

The relationship between pH and pK_a can easily be derived from Eqs. (3) and (4): the pH equation can be represented by:

$$\text{pH} = pK_a + \log \frac{A^-}{HA} + \log \gamma_{A^-} \tag{5}$$

for acids and

$$\text{pH} = pK_a - \log \frac{HA}{A^-} - \log \gamma_{A^-} \tag{5'}$$

for bases

The activity coefficients can be calculated from Debye–Hückel's theory at 25°C with:

$$\log \gamma = \frac{0.5085 \cdot Z^2 \cdot \sqrt{\mu}}{1 + 0.3281 \cdot a \cdot \sqrt{\mu}} \tag{6}$$

with

$$\mu = 1/2 \sum_{i=1} C_i \cdot Z_i^2$$

Where 'a' is the hydrated diameter of an ion in Å, C is the molarity of the ion, Z is the valency of the ion and μ is the ionic strength of the solution.

In general, the exact value of 'a' ranges from 1 to 11. As it cannot be determined easily, a value of 5 can be adopted.

The substitution of the activity coefficient in the pH Eqs. (5) and (5′) will give:

$$\mathrm{pH} = \mathrm{p}K_a + \log\frac{[A^-]}{[HA]} \pm \frac{0.5085 \cdot Z^2 \cdot \sqrt{\mu}}{1 + 0.3281 \cdot a \cdot \sqrt{\mu}} \quad (7)$$

With the activity coefficient being positive for acids and negative for bases.

2.2. Relationship between mobility and pK_a

The relationship between electrophoretic mobility and $\mathrm{p}K_a$ can be described as follows: mobility (M_e) is a function of the field strength (E) in the capillary (which is dependent on the applied voltage (V) and the total capillary length (L_c)), as well as the electrophoretic velocity (υ_e).

$$M_e = \frac{\upsilon_e}{E} \text{ with } \upsilon_e = \frac{\upsilon_e}{t} \text{ and } E = \frac{V}{L_c} \quad (8)$$

If the distance between the injection point and the detector is L_d and the migration time of an analyte is t_{app}, the apparent mobility (M_{app}) can be expressed as:

$$M_{app} = \frac{\upsilon_{app}}{E} = \frac{L_c L_d}{t_{app} \cdot V} \quad (9)$$

M_{app} is not equal to M_e because of the influence of the electroosmotic flow (EOF) generated by the capillary wall as a function of pH.

$$M_e = M_{app} - M_{EOF} \quad (10)$$

The EOF can easily be determined by using a marker that remains neutral along the pH range selected for the different buffers. The mobility of an ion can, therefore, be related to:

$$M_e = \frac{L_c L_d}{V}\left(\frac{1}{t_{app}} - \frac{1}{t_{EOF}}\right) \quad (11)$$

For example, when a neutral acid (HA) is deprotonated the net electrophoretic mobility M_e in a selected buffer is given by:

$$M_e = \alpha M_a \quad (12)$$

M_a is the electrophoretic mobility of the fully deprotonated species A^-, and alpha is the fraction of analyte ionised.

Using this relation, it is possible to rewrite the ratio A^-/HA in terms of mobility by substitution of α

$$\frac{[A^-]}{[HA]} = \frac{\alpha}{1-\alpha} = \frac{M_e}{M_a - M_e} \quad (13)$$

By substitution in pH Eq. (7)

$$\mathrm{p}K_a^{th} = \mathrm{pH} - \log\left(\frac{M_e}{M_a - M_e}\right) + \frac{0.5085 \cdot Z^2 \cdot \sqrt{\mu}}{1 + 0.3281 \cdot a \cdot \sqrt{\mu}} \quad (14)$$

Finally, for diluted solutions, we can obtain the relationship between $\mathrm{p}K_a$, pH and the mobilities of fully charged (M_a) and partially charged (M_e) species:

$$\mathrm{p}K_a = \mathrm{pH} - \log\left(\frac{M_e}{M_a - M_e}\right) \quad (15)$$

Eq. (13) can be transformed into a regression model and electrophoretic mobility can, therefore, be expressed as a function of $\mathrm{p}K_a$ and M_a or M_b by:

$$M_e = \frac{M_a}{10^{(\mathrm{p}k_a - \mathrm{pH})} + 1} \quad (16)$$

for acid and

$$M_e = \frac{M_b}{10^{(\mathrm{pH} - \mathrm{p}K_a)} + 1} \quad (17)$$

for bases

Experimentally, the $\mathrm{p}K_a$ can be calculated on the basis of measurement of retention times of the species and the neutral marker, for a series of buffers at different pH. The electrophoretic mobilities (M_e), calculated using Eq. (11) and plotted against the pH, can be fitted with the sigmoidal model represented by Eqs. (16) and (17) using a non-linear regression [4]. The two unknowns mobility of the fully ionised species (M_a) and $\mathrm{p}K_a$ are the regression parameters.

3. Material and methods

3.1. Chemicals and reagents

All API used as test analytes were synthesised by Eli Lilly and Company (Indianapolis, USA).

Table 1
Buffer preparation method (from [5])

pH range	Buffer	Stock solution[a]	Ionic strength
2.7–3.3	Phosphate	1 M H_3PO_4 1 M NaH_2PO_4	0.05
3.4–5.4	Acetate	1 M CH_3COOH 1 M CH_3COONa	0.05
5.7–8.0	Phosphate	0.1 M NaH_2PO_4 0.1 M Na_2HPO_4	0.05
7.5–9.2	Borate	0.1 M $Na_2B_4O_7$ 0.4 M H_3BO_3	0.05
9.2–12.1	Borate	0.1 M $Na_2B_4O_7$ 0.1 N NaOH	0.05

[a] Stock solutions were mixed, diluted to $\mu = 0.05$ and adjusted to appropriate pH.

Reagents used for preparing the different buffers were all of p.a. quality grade from Merck (Darmstad, Germany).

Methanol was of chromatographic grade from Acros (NJ, USA). Water used in all experiments was of Milli-Q quality from Millipore (Bedford, USA). Mesityl oxide used as neutral marker was from Sigma (St. Louis, USA).

3.2. Instrumental parameters

Two different systems were used for the experiments: a SPECTRA PHORESIS 1000 (Spectra Physics Inc. San Jose, CA) and a BECKMAN P/ACE system 5500 (Beckman Instruments Inc., Fullerton, CA).

The analytes were detected by UV absorbance at 214 nm. The uncoated capillary was a fused silica capillary (Composite Metal Services LTD, UK)), 37 cm × 75 μm ID, with the detector window at 29 cm from the injector. The samples were injected for 5 s by hydrodynamic mode at 30°C. The capillary was operated at 15 kV with the current not exceeding 50 μA. The electrophorograms and data were recorded by a Millennium32 data acquisition System (Waters, Milford, MA).

In order to equilibrate the column and to minimize hysteresis effects, the capillary was rinsed prior to each run in the following sequence: 2.5 min with NaOH 0.1 N, 2.5 min with water and 3 min with running buffer.

The potentiometric system consisted of a Sirius titrator GlpKa from Sirius Analytical Instrument Ltd (UK) with combined electrode; the GlpKa instrument is supplied with Sirius pK_a log P software for the calculation of pK_a.

The computational predictions were made using ACD/pK_a software from Advanced Chemistry Development Inc. (UK).

3.3. Buffer preparation

The buffers were prepared by mixing two stock solutions as described in Table 1. The final solutions were diluted to a final ionic strength of 0.05 M. Buffer pHs were measured using a Knick Portamess Calimatic 752 pH meter. The buffers

Table 2
List of compounds investigated and comparison of methods

Compound	Computational prediction	CE	Sirius titration
LY329632	10.49	10.64	10.16
LY334370	9.58	9.92	9.57
LY397584	9.33	9.70	9.53
	6.50	4.18	4.42
LY432509	8.34	6.93	No result (compound is insoluble)
LY389371	10.42	9.96	9.53
LY389372	10.44	10.01	9.57
LY393558	7.48	6.77	6.65
CPD A	4.76	4.45	4.16
CPD B	4.77	4.57	4.28

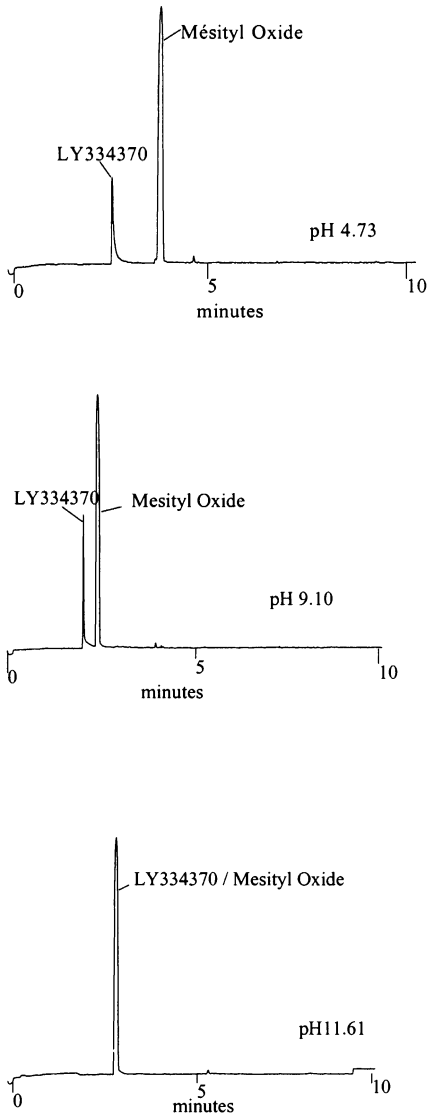

Fig. 1. Electrophorogram at different pH; 4.73, 9.10 and 11.61 for LY334370 and mesityl oxide. Conditions, capillary 37cm × 75 μm i.d. (29 cm to the detector); separation solutions, buffers listed in Table 1; field strength, 15 kV; temperature, 30°C; Hydrodynamic injection 5 s; detection 214 nm; compound LY334370.

were filtered through a Millex® HA 0.2 μm filter (Millipore, Bedford, USA).

3.4. Sample preparation

Sample solutions were prepared at a concentration of 50 μg/ml in water. Mesityl oxide (1 mg/ml) was used as a neutral marker for the calculation of the effective mobilities.

Mesityl oxide gives a high absorbance and peak symmetry. In the event of aqueous insolubility the compounds were dissolved in methanol and diluted to volume with water. The samples were filtered through a Millex® HA 0.2 μm filter.

4. Results and discussion

Results and discussion are presented below with an example of outputs (electrophorograms, titration curves), and a review of the main parameters affecting the determination of pK_a values.

Electrophorograms obtained for different pH (pH 4.73, 9.10 and 11.61, respectively) values for both the neutral marker and the compound analysed are represented in Fig. 1.

When the electrophoretic mobility calculated at different pH for a single analyte is represented as a function of pH, plots shown in Figs. 2 and 3 are obtained for a monovalent and a divalent compound, respectively. The pK_a can be graphically determined at the inflexion point of the sigmoidal curve. The value can also be calculated by using a non-linear regression model on the experimental pair values of pH and M_e as described in the calculation section below.

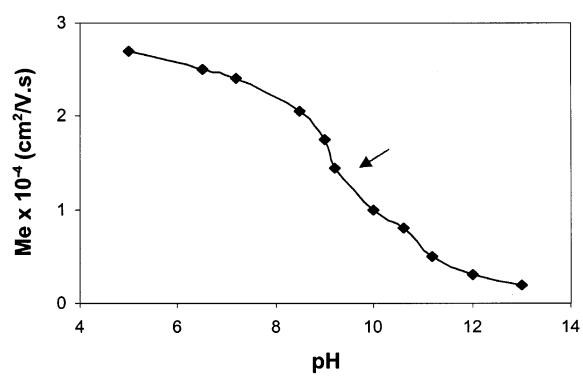

Fig. 2. Dependence of the effective mobilities of a monovalent compound (LY334370) on pH. Conditions as given in Fig. 1. Arrow indicates the pH equal to the pK_a.

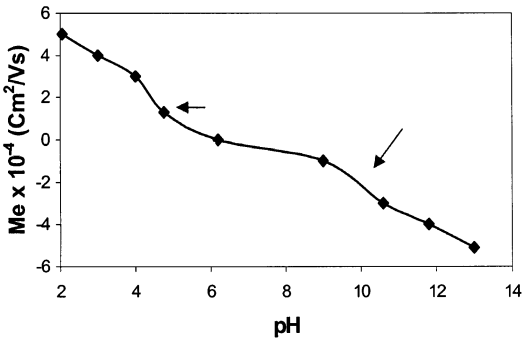

Fig. 3. Dependence of the effective mobilities of a divalent compound (LY397584) on pH. Conditions as given in Fig. 1. Arrows indicate the pHs equal to the pK_as.

4.1. Choice of buffers

A wide pH range has been covered in this experimental work. This has been possible thanks to the use of several types of buffers; to compromise between the EOF and the analysis time, a constant ionic strength of 0.05 was chosen for all buffers.

Our choice was also motivated by the desire to avoid the influence of dissolved CO_2 on the final pH. The buffer concentrations were high enough to allow for stacking in order to improve the sensitivity of the method.

4.2. Influence of the EOF marker

It is essential to use an appropriate EOF marker in order to determine the effective mobility from the apparent mobility. Mesytil oxide was chosen as a neutral marker from among others such as acetone, phenol, methanol, and dimethylsulfoxide. The importance of an appropriate marker, which is not affected by the background electrolyte, has been shown [6].

This can easily be verified by checking the correspondence of the migration time of the water dip (UV signal difference between sample and background electrolyte) prior to experimentation.

The same author also stressed the importance of the capillary surface during the experiment. Our rinsing procedure has proved to give a good inter-run comparison of data.

4.3. Comparison of pK_a values generated with different methods

All compounds tested are listed with their pK_a values obtained by the different approach in Table 2.

As shown in Figs. 4 and 5 a good correlation was found between the CE and Sirius methods as well as an acceptable correlation between the CE and computer calculations.

The lowest correlation between CE and computational values may be explained by inherent limitations of the prediction software, such as incorrect modelling of the molecular fragments or difficulty in handling compounds with multiple ionisable functions.

The strong interest in capillary electrophoresis is due to the fact that for impure compound pK_a

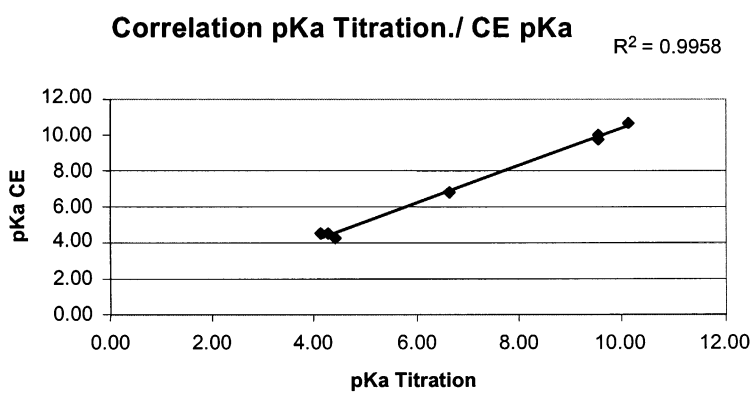

Fig. 4. Correlation between pK_a determined by titration and pK_a determined by CE.

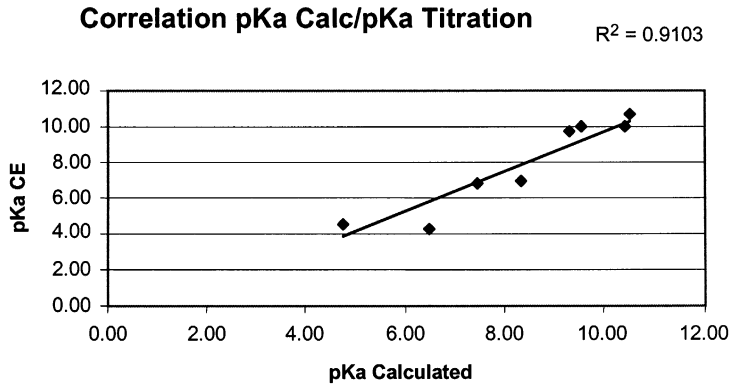

Fig. 5. Correlation between pK_a calculated (computational method) and pK_a determined by CE.

determination is not affected by the purity or the solubility [8] of the compound. Indeed, Table 3 shows that potentiometric titration was impossible for the least soluble compound (LY432509) and, therefore, CE was the ideal technique.

Some of the differences observed between the pK_a determination by CE and other approaches can also be explained by the fact that the activity coefficients were neglected for the calculations even though the ionic strength was 0.05.

4.4. Precision of the data

Data presented in Tables 3 and 4 show that a good reproducibility can be obtained from day to day for the determination of pK_as. With the current procedure and equipment, all data generated were within 2–5% of the mean value. In general, the precision should be expected to be better at lower pH while the influence of EOF is reduced and, therefore, the discrimination effect between the apparent mobilities of the cations is higher.

4.5. Choice of the calculation method

As already shown [3], the use of non-linear regression is the simplest and most precise way (with less replicates) to obtain pK_a values of new API by CE.

With this approach, a wide range of pH can be covered by using a series of equally spaced pH buffers which are linear with respect to the fraction of ionisation of the evaluated compound.

The following equation (i.e. for bases):

$$M_e = \frac{M_a}{10^{(pH - pK_a)} + 1}$$

can easily be resolved by using a simple statistical software (e.g. JMP® from SAS Institute Inc).

Table 3
Repeatability of determinations for a monovalent compound: LY334370

LY334370HCl	pK_a
	9.66
	9.94
	10.01
	9.36
	9.84
Mean	9.76
CV%	2.7

Table 4
Repeatability of determinations for a divalent compound: LY397584

LY397584	pK_a 1	pK_a 2
	9.78	4.09
	9.97	4.02
	9.96	4.06
	9.38	4.02
	9.70	4.05
Mean	9.76	4.05
CV%	2.5	0.7

The values of both parameters pK_a and M_a (absolute mobility), can be obtained (see Figs. 2 and 3) from the calculation of the non-linear regression.

As an alternative method of calculation, the linear model also gives a good correlation [7]. Estimations using this model suffer from higher variances and some discrepancies can therefore be found in the calculated data.

For multivalent pK_a determination (i.e. compound LY397584), rather than using sophisticated formula the same non-linear model was run twice on the two sets of pH data bracketing each inflexion point on the curve represented in Fig. 3.

5. Conclusions

At the beginning of development of a new drug substance, when still in the lead optimisation phase, for compounds that are not always pure or soluble, CE has proved to be a suitable and useful technique for the determination of pK_as. The technique can rapidly be fully automated and has also proved to be effective when using small quantities of material (< 2 mg).

An estimation of the accuracy of the pK_a values on basic compounds, when compared with other techniques, is around 0.5 pK_a units, which is acceptable during this early screening phase.

The data generated using the CE method were comparable to those obtained with the titration method (Sirius) or with the data calculated using the ACD/pK_a software.

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