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Determination of pK_a values of benzimidazole derivatives from mobility obtained by capillary electrophoresis

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ABSTRACT

Dissociation constants of benzimidazole derivatives have been determined using capillary zone electrophoresis (CZE). Since CZE is a separation method, high purity and known concentration for the samples is not necessary because only mobilities are measured. The precision of pK_a measurements of seven compounds is useful to observe pK_a shifts induced by chemical variations. Some of them were compared to potentiometry and spectroscopy experiments. Good correlated pK_a values are observed between the three analytical techniques.

1. Introduction

The knowledge of dissociation constant is a key parameter for understanding chemical interaction of the interest compound and its pharmacological target. Relationships between acidity constant, K_a and structure may prove useful in drug design studies and in explaining the biopharmaceutical properties of substances like solubility, absorption, distribution, metabolism and elimination. Many biologically active molecules are fully or partially ionized at physiological pH, and it has often been shown that the presence of charged groups is necessary for biological activity and/or solubility. Hence the discovery of new molecules requires accurate determination of K_a values.

Capillary electrophoresis (CE), has been introduced as a technique for convenient and precise aqueous pK_a determination [1,2]. The method relies on the principle that the solute exhibits an electrophoretic mobility continuum *vs* pH. In its uncharged state, the solute has no mobility and in its fully ionized state, it has a maximum mobility. Intermediate mobilities are a function of the dissociation equilibrium and can be solved by regression analysis [3]. This method offers several advantages such as it requires small amounts of sample at low solute concentrations, the procedure do not require measurement of solute or titrant concentrations but only migration times. Calculation is straightforward and independent of the solute purity and is a universal technique for determining pK_a values over a wide pH range [3]. As reported by different studies, CE is a method of choice compared to multiwavelength spectrophotometric or potentiometric techniques [4,5,18].

The aim of this work is to determine the dissociation constants of a series of new benzimidazoles derivatives using CE with direct UV detection and some of these values were confirmed by potentiometric and UV spectrophotometric methods. Those new compounds were designed starting from the lead compound **1** (S27847, Fig. 1): firstly, by modification of the cyclohexylphenyl moiety and secondly, by introducing diversity on the aromatic moiety of the benzimidazole ring to obtain potential therapeutic agents. Many of them present high *in vitro* activation of the AMP-kinase on fresh rat hepatocytes. AMPK itself plays a key role in the regulation of metabolism within the muscle cell and has been already identified as a potential target for type 2 diabetes mellitus and obesity [6–9].

2. Experimental

2.1. Instrumentation

Capillary electrophoretic experiments were performed using a Beckman P/ACE MDQ series Capillary Electrophoresis System with diode-array detector from 190 nm to 600 nm and system 32 Karat version 4.0 software (Beckman Instruments, Villepinte, France). The CE separations were all done with the conventional operating (anodic injection). An uncoated fused-silica CE column [50.2 cm

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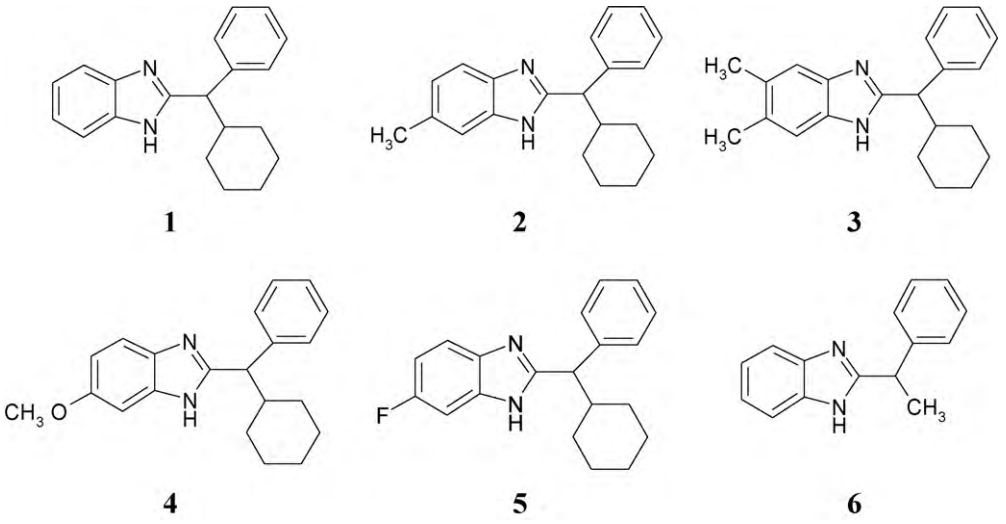

Fig. 1. Chemical structures of compounds **1**–**6**.

(40 cm to detector) × 50 μm I.D.] was obtained from Composite Metal Services Ltd. (UK). The temperature of the capillary was maintained at a fixed level (25 °C) by means of a liquid coolant in the capillary cartridge.

New capillaries were rinsed with 0.1 M NaOH for 2 min, water for 5 min and the running electrolyte for 20 min. Between runs the capillaries were washed with 0.1 M NaOH for 2 min, water for 2 min and equilibrated by flushing with the running electrolyte for 5 min. All injections were done in the hydrodynamic mode (5 s, 1.0 psi or 6.7 kPa). The capillary was operated at 20 kV (0.40 kV/cm) with the current not exceeding 50 μA. The detection wavelengths were 205, 220 and 270 nm. Six replicate injections were performed for each compounds and electrophoretic mobilities were used in the calculation of pK_a.

Micro-scale potentiometric titrations with computer-aided evaluation of the dissociation constants have been performed using the Sirius PCA-200 instrument (Sirius Analytical Instrument, Ltd., Forest Row, East Sussex, UK) with adjacent computer-aided evaluation of pK_a values (Sirius RefinementPro software using pK_aLogP program). The standardization of the electrode system was carried out every day by a two steps standardization procedure as follows. First, electrode calibration consisted of performing an aqueous blank titration and computer-aided evaluation of the "Four Plus™" parameters. Second, base (0.5 M KOH) titrant solution is standardized by titration with potassium hydrogen phtalate (mass weighted exactly from 0.1500 to 0.1900 g and dissolved precisely in 20 mL water). The titration curves were interpreted by means of the program p*K*aLogP.

In aqueous solution the four-parameter equation has been used for this purpose

$$\mathrm{pH} = \alpha + S(-\log[\mathrm{H}^+]) + j_{\mathrm{H}}[\mathrm{H}^+] + j_{\mathrm{OH}}K_W/[\mathrm{H}^+] \quad (1)$$

The parameters are determined by a weighted nonlinear least-squares procedure. The intercept parameter α in aqueous solution corresponds mainly to the negative logarithm of the activity coefficient of H_3O^+ at the working temperature and ionic strength. S stands for the Nernst slope. The j_H term corrects pH readings for the nonlinear pH response due to liquid junction and asymmetry potentials in acidic solutions (pH 1.5–2.5), while the j_{OH} term corrects for high-pH (pH > 11) non-linear effects [10].

The UV–visible spectra, obtained to determine absorptiometric p*K*a values, were recorded at each pH using a Kontron UV–Vis spectrometer (Uvikon Instruments, Trappes, France) equipped with 1 cm path length cell.

The pH of the buffer solutions used for CE and UV experiments was measured using a combination pH electrode (Hanna Instruments, Rhode Island, USA).

2.2. Chemicals and reagents

Benzimidazoles derivatives **1**–**6** used in this work, were synthesized as followed [11]. Boric acid and sodium borate were obtained from Sigma (Saint Quentin Fallavier, France). The other reagents used in the experiments were all of analytical grade and all buffers were prepared with water, with a resistivity of 18.2 MΩ cm from a milli-Q water purification system (Veolia Water, STI, Le Plessis Robinson, France).

2.3. Procedures

2.3.1. CE experiments

For CE experiments, Stock solutions of samples were prepared in ethanol (2.5 mM) and diluted to 0.25 mM with ethanol and with a 1% DMSO solution to obtain a final solution with 0.1% in neutral marker and 40% in alcohol. The solutions were always degassed in an ultrasonic bath prior to use.

Buffer solutions [12–14] of different pH values (Table 1) were obtained as followed: phosphate buffer solutions were prepared by mixing appropriate volumes of stock solutions (0.1 M NaH_2PO_4 and 0.1 M H_3PO_4 for pH range from 2.38 to 4.23) or (0.1 M Na_2HPO_4 and 0.1 M NaH_2PO_4 for pH range from 5.93 to 8.04) and subsequently diluting with water to yield an ionic strength of 0.01; acetate buffer solutions were prepared by mixing appropriate volumes of stock solutions (0.1 M CH_3COONa and 0.1 M CH_3COOH for pH range from 3.77 to 5.96) and subsequently diluting with water to yield an ionic strength of 0.01. The buffers were first filtered through a 0.45 μm filter and degassed in an ultrasonic bath prior to use. Buffer solutions were tested for stability of the pH value. Elec-

Table 1
pK_a values of compounds **1**–**6** determined by CE experiments.

Compound	pK_a	RSD %
1	5.24 ± 0.06	1.2
2	4.60 ± 0.07	1.4
3	4.70 ± 0.03	0.5
4	5.32 ± 0.07	1.4
5	4.69 ± 0.01	0.1
6	5.55 ± 0.01	0.1

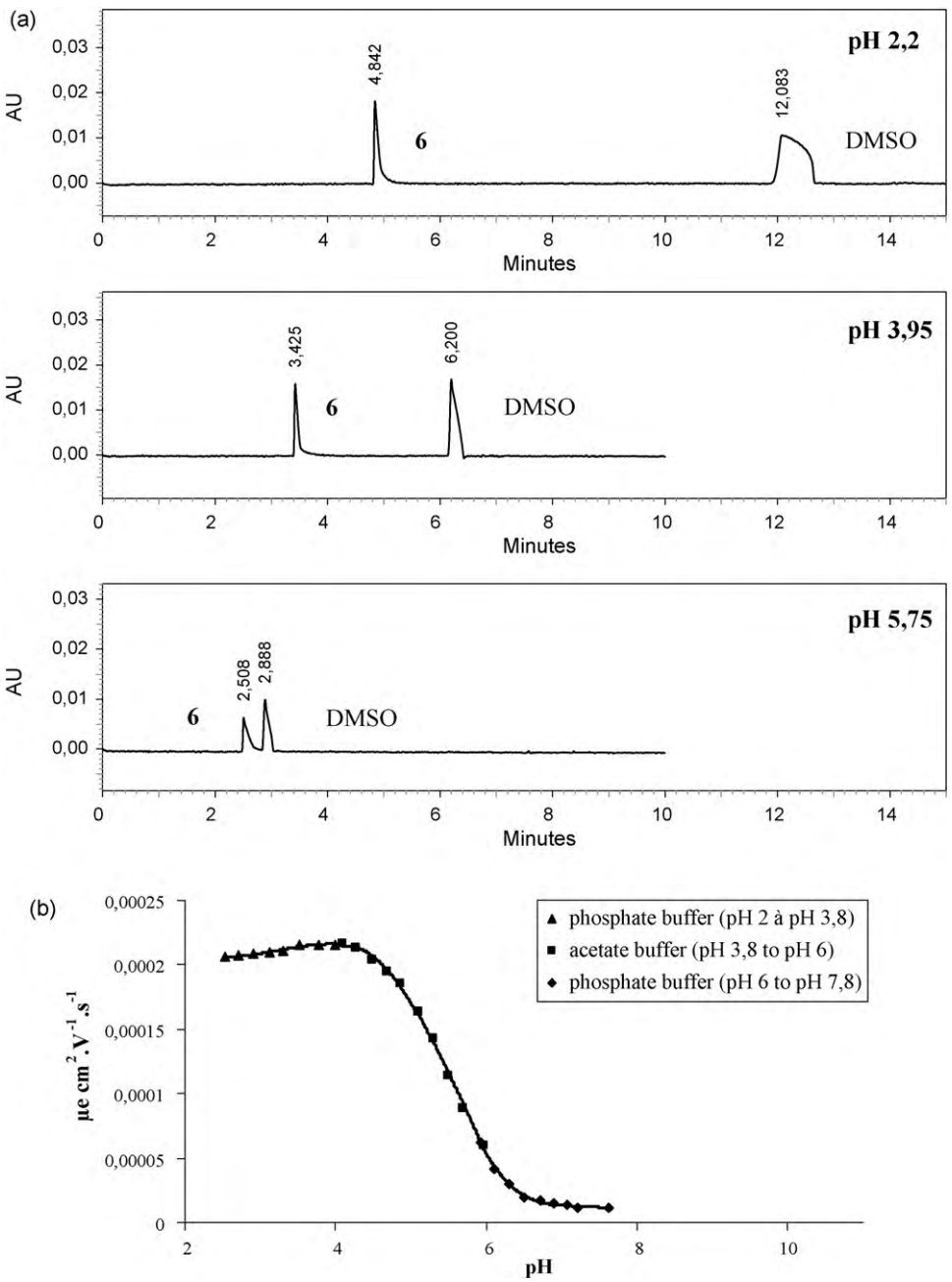

Fig. 2. (a) Stacked electropherograms showing electrophoretic mobility variations of compound **6** and DMSO (electroosmotic flow marker) for pH values of 2.2; 3.95 and 5.75 (□ = 205 nm). (b) Influence of the pH of the electrolyte solution on the effective mobility of compound **6**.

trolyse was observed at both cathode and anode, with alcalinization and acidification of the buffer solutions, respectively. This drawback is prevented by the replacement of the buffer solutions every 10 runs. All the benzimidazoles derivatives **1–6** used in this work, have one relevant ionizable functional group, the protonation of the nitrogen atom of the heterocycle. They are basic compounds, monobase B, where the acid–base dissociation constant K_a is defined as:

$$BH^+ \overset{K_a}{\rightleftarrows} B + H^+ \quad K_a = \frac{[B](H^+)}{\gamma_{BH^+}[BH^+]} \tag{2}$$

where (H^+) is the activity of the protons, [B] and $[BH^+]$ are the concentrations of neutral and protonated forms and γ_{BH^+} is the activity coefficient of ionized species.

$$\frac{1}{\mu_e} = K_a \frac{\gamma_{BH^+}}{\mu_{BH^+}(H^+)} + \frac{1}{\mu_{BH^+}} \tag{3}$$

where μ_e is the electrophoretic mobility of the analyte and μ_{BH^+} the electrophoretic mobility of the fully protonated molecule.

The K_a and the μ_{BH^+} values are directly obtained from the calculated slope and the ratio (1/intercept). The activity coefficient, γ_{BH^+}, calculated from Debye–Hückel's theory, in our experiments, was considered as constant (calculated value 0.89 for the ionic strength 0.01).

2.3.2. Potentiometric experiments

For potentiometric measurements, solutions of individual benzimidazole derivatives were prepared at a concentration of 10^{-4} M,

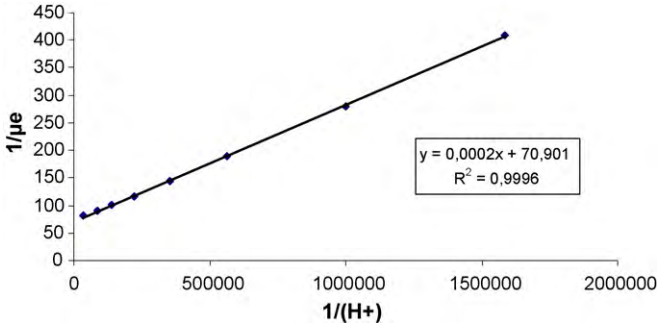

Fig. 3. Graphical determination of the pK_a value of compound **6**.

in aqueous 0.154 M potassium chloride to adjust the ionic strength (I = 0.154). The pH was lowered to 1.8 using 0.5 M HCl before titration which was done with 0.5 M KOH up to pH 12.2. Five measurements were carried out.

2.3.3. Spectrophotometric measurements

Spectrophotometric measurements were carried out with solutions of individual benzimidazole derivatives prepared at a final concentration of 5×10^{-6} M. As used in CE experiments, same buffer solutions, with constant ionic strength (I = 0.01), are chosen for UV measurements. The spectra of solutions were recorded in a wavelength range of 200–400 nm. Determination of pK_a was performed at two-extracted wavelength for five independent experiments.

3. Results and discussion

3.1. Choice of buffer pH range

To measure the pK_a values of the homogeneous chemical class described here, a pH range comprised between 2 and 8.2 has been used. The phosphate buffer at pH 2 allowed to measure the maximum electrophoretic mobility of the fully ionized compounds. To control the reproducibility of the μ_e, six injections for each monobase were run. The relative standard deviation (CV%) of the effective mobilities calculated for all compounds were under 2%.

3.2. Choice of buffer concentrations

As previously described by Matoga et al. [1], ideal electrophoretic conditions are a compromise between a constant ionic strength (obtained with high buffer concentration to allow a better stability of the buffer solution pH) and low Joule heating (minimum effect with diluted buffers). We have chosen to privilege weak concentration to obtain I = 0.01 which induces low current intensity with the Joule effect counterbalanced by a cooling refrigerant to maintained a constant temperature. Ohm's law was verified (linear relationships between current and voltage) and showed no excessive Joule heating generated during the electrophoresis process.

3.3. Calculation of pK_a values

In the present paper we studied the determination of the dissociation constant of benzimidazole derivatives by capillary zone electrophoresis. Electropherograms shown in Fig. 2a are obtained for the mono-base **6** at various pH. The pK_a can be graphically determined at the inflexion point of the sigmoidal curve (Fig. 2b). For precise determination of pK_a values, we used the linear regression analysis on the data according to Eq. (3). An example of linear regression with benzimidazole derivative **6** is given in Fig. 3. As for all compounds, the correlation coefficient values, r, for the linear fit, was greater than 0.999. Slope and intercept values for compound **6** are $2.0 \times 10^{-4} \pm 0.11 \times 10^{-4}$ and 70.9 ± 6.9, respectively, calculated on six experiments. All pK_a values calculated from the slopes (K_a) are given in Table 1. The pK_a values of the derivatives **1** and **2** obtained by CZE are close to those determined by UV spectroscopy and titration. The values are respectively for **1** (5.24; 5.21; 5.25) and **2** (4.60; 4.57; 4.67). Good agreement was recently reported with similar molecules [15,16].

In the present study, significant differences of pK_a values are observed for benzimidazoles with electron–donor substituent placed on the aromatic ring. A direct comparison of the pK_a values of compounds **1** on one hand and **2**, **3**, **5** on the other hand, demonstrated the acidity increase of compounds with methyl group or fluorine atom as substituent of the aromatic ring in the benzimidazole series. The number of substituents seems not to influence the values of pK_a. Replacement with methoxy group as substituent on the aromatic ring (compounds **1** *vs* **4**) leads to small enhancement of the basicity (pK_a = 5.24 and 5.32, respectively). The modification of the cyclohexylphenyl moiety at the asymmetric carbon by a methyl group leads to basicity increase (compounds **1** *vs* **6**).

4. Conclusions

In this investigation, the determination of dissociation constants of six substituted benzimidazole derivatives has been performed. The proposed CZE method has been proven to be convenient to determine the pK_a values of these compounds with good measurement precisions. Basic character of different compounds is dependent of adjacent aromatic groups. An electron–acceptor cyano function substituted in para position of the benzyl ring, led to a decrease of the pK_a value. Modifications performed on the other adjacent ring, the heterocycle, did not affect the pK_a of the imidazole. Finally, this study revealed a good correlation between the CZE and the spectroscopic and potentiometric methods; Moreover, CZE allowed to work with very weak quantities of compounds Acidity constants knowledge of new compounds is an essential goal for further analytical studies such as enantiomeric separation in aqueous solutions (generally performed by CE) or affinity constant determinations (by CE or by spectroscopic methods as NMR or fluorescence). We would perform CE methods for enantiomeric separations and affinity constant determinations of our compounds with neutral or anionic cyclodextrines at compatible pH range for fully ionized forms [17].

References

[1] M. Matoga, E. Laborde-Kummer, M.H. Langlois, P. Dallet, J.J. Bosc, C. Jarry, J.P. Dubost, Determination of pKa values of 2-amino-2-oxazolines by capillary electrophoresis, J. Chromatogr. A 984 (2003) 253–260.
[2] S.M.C. Buckenmaier, D.V. McCalley, M.R. Euerby, Determination of pK values of organic bases in aqueous acetonitrile solutions using capillary electrophoresis, J. Chromatogr. A 1004 (2003) 71–79.
[3] S.J. Gluck, J.A. Cleveland Jr., Capillary zone electrophoresis for the determination of dissociation constants, J. Chromatogr. A 680 (1994) 43–48.
[4] G. Popovic, L. Milovanovic, V. Kapetanovic, Study of acid–base equilibria of fleroxacin, J. Pharm. Biomed. Anal. 18 (1998) 859–863.
[5] E. Jiménez-Lozano, I. Marqués, D. Barrón, J.L. Beltrán, J. Barbosa, Determination of pKa values of quinolones from mobility and spectroscopic data obtained by capillary electrophoresis and a diode array detector, Anal. Chim. Acta 464 (2002) 37–45.
[6] W.W. Winder, D.G. Hardie, AMP-activated protein kinase, a metabolic master switch: possible roles in type 2 diabetes, Am. J. Physiol. 277 (1999) E1–E10.
[7] D.E. Moller, New drug targets for type 2 diabetes and the metabolic syndrome, Nature 414 (2001) 821–827.
[8] Y. Minokoshi, Y.B. Kim, O.D. Peroni, L.G. Fryer, C. Muller, D. Carling, B.B. Kahn, Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase, Nature 415 (2002) 339–343.
[9] N. Ruderman, M. Pentki, AMP kinase and malonyl-CoA: targets for therapy of the metabolic syndrome, Nat. Rev. Drug. Discov. 3 (2004) 340–351.
[10] C. Kahle, U. Holzgrabe, Chirality 16 (2004) 509–514.

[11] J. Charton, S. Girault-Mizzi, M.A. Debreu-Fontaine, F. Foufelle, I. Hainault, J.G. Bizot-Espiard, D.H. Caignard, C. Sergheraert, Synthesis and biological evaluation of benzimidazole derivatives as potent AMP-activated ptotein kinase activators, Biol. Med. Chem. 14 (2006) 4490–4518.
[12] S.K. Poole, S. Patel, K. Dehring, H. Workman, C.F. Poole, Determination of acid dissociation constants by capillary electrophoresis, J. Chromatogr. A 1037 (2004) 445–454.
[13] Z. Jia, Physicochemical profiling by capillary electrophoresis, Curr. Pharm. Anal. 1 (2005) 45–56.
[14] Z. Jia, T. Ramstad, M. Zhong, Medium-throughput p*K*a screening of pharmaceuticals by pressure-assisted capillary electrophoresis, Electrophoresis 22 (2001) 1112–1118.
[15] G. Jerez, G. Kaufman, M. Prystai, S. Schenkeveld, K.K. Donkor, Determination of thermodynamic pK_a values of benzimidazole and benzimidazole derivatives by capillary electrophoresis, J. Sep. Sci. 32 (2009) 1087–1095.
[16] C.F. Poole, Capillary-Electromigration Separation Techniques in the Essence of Chromatography, Elsevier Sciences B.V. Ed., Amsterdam, The Nerderlands, 2003, pp. 658–717.
[17] E. Lipka, J. Charton, M.P. Vaccher, M. Folly-Klan, J.P. Bonte, C. Vaccher, Enantioseparation of chiral benzimidazole derivatives by electrokinetic chromatography using sulfated cyclodextrins, J. Sep. Sci. 32 (2009) 1907–1915.
[18] C. Foulon, N. Duhal, B. Lacroix-Callens, C. Vaccher, J.P. Bonte, J.F. Goossens, Determination of pK_a values of benzoxa-, benzothia- and benzoselena-zolinone derivatives from mobility obtained by capillary electrophoresis. Comparison with potentiometric titration and spectroscopic data, Eur. J. Pharm. Sci. 31 (2007) 165–171.