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Determination of ionization constants of *N*-imidazole derivatives, aromatase inhibitors, using capillary electrophoresis and influence of substituents on pK_a shifts

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Abstract

Capillary electrophoresis (CE) was used as a method to determine the acidity constants of eight aromatase inhibitors. This method was validated by comparison of results obtained with a traditional method, UV spectroscopy, and additionally with computational calculations. We confirmed here, with our series of compounds, that capillary electrophoresis is an attractive method for pK_a measurements which is based on migration time or mobilities of the ionic species over a range of pH values. The precision of pK_a measurements of *N*-imidazole derivatives is useful to observe pK_a shifts induced by chemical modifications introduced on adjacent aromatic rings such as heterocycle (benzoxa- or benzothiazolinone) or substituted benzyle. The knowledge of these pK_a values is a great interest to predict migration of solutes and qualitative interactions with ionized cyclodextrines as chiral selectors in further enantioseparative CE studies.

Keywords: Ionization constants; Dissociation constants; Imidazoles; Enzyme inhibitors; Benzoxazolinones; Benzothiazolinones

1. Introduction

Compounds such as vorozole, anastrozole and more recently letrozole (Fig. 1) are useful in second-line therapy of oestrogen-dependent breast cancer in postmenopausal women [1]. Six benzoxazolinone and two benzothiazolinone derivatives with adjacent imidazole moiety have been designed as P-450 aromatase inhibitors on the basis of pharmacophore model developed by Auvray and co-workers (Table 1) [2,3]. In vitro activities of many of them were higher than fadrozole one's, the lead compound of this series (Fig. 1). It is noteworthy that there is a great interest to develop this class of therapeutic agents for treatment of breast tumour [4]. For fadrozole, the eutomer is the (*S*)-form. Since our compounds possess stereogenic centres (Fig. 2), we described recently the chiral resolution of benzoxa- and benzothiazolinone by high-performance liquid chromatography (HPLC) using derivatized cellulose and amylose chiral stationary phases [5]. In the continuity of our work on the enantioseparation of biologically active racemates, we performed the same experiments by capillary electrophoresis (CE) using native or substituted cyclodextrin (CD) as additive chiral selector in the background electrolyte [6]. For developing separative CE methods, the ionization constant of solute has become of great importance especially when using an ionized chiral selector to predict the migration of solute and to perform the enantioseparation [6]. Additionally, studies of selector–selectand interactions such as stoichiometry measurements of complexes or binding constant determinations, could be performed using NMR spectroscopy, electrospray ionization mass spectrometry or spectrofluorimetry [7,8]. Moreover, the knowledge of ionization constants is an important challenge for the understanding of some chemical phenomena such as biological uptake, pharmacological activity or in vivo biodisponibility. Hence, the discovery of new molecules requires accurate determination of pK_a values.

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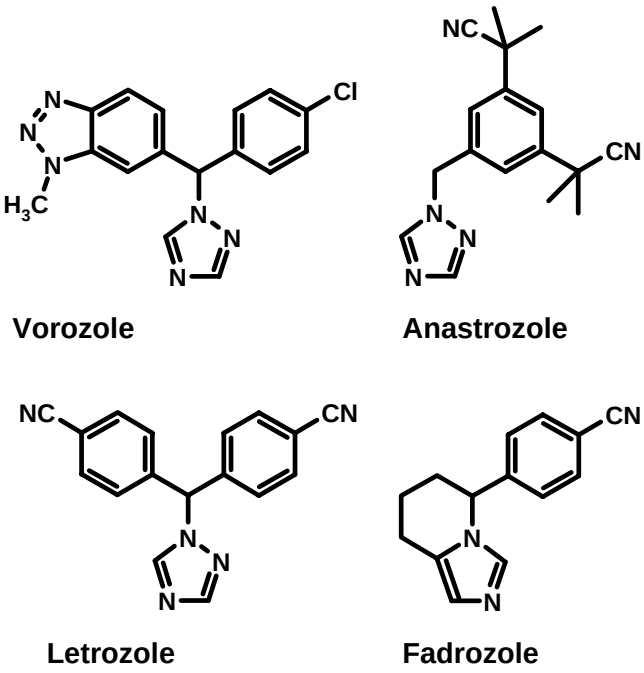

Fig. 1. Non-steroidal aromatase inhibitors.

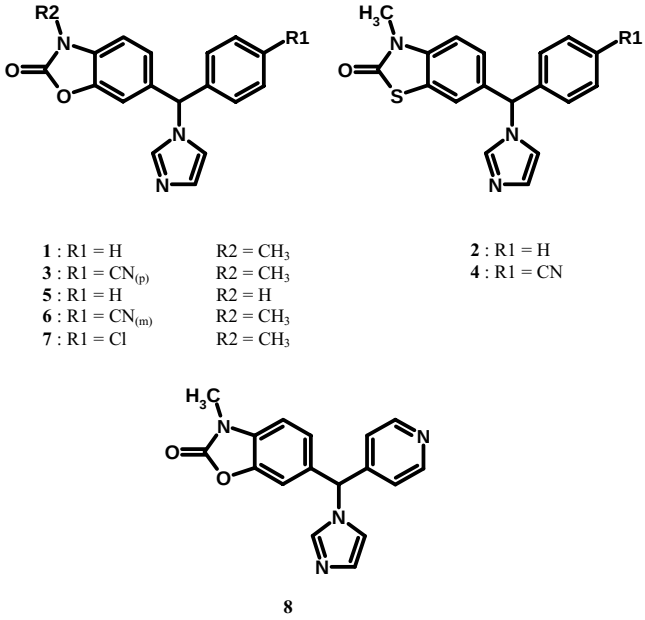

Fig. 2. Chemical structures of the *N*-imidazole derivatives studied.

Recently, CE has been used as a convenient method for precise measurements of ionization constants in aqueous by Matoga et al. [9] and in aqueous–organic buffers by Buckenmaier et al. [10]. Several advantages of CE methods versus the classical pK_a determinations (potentiometric and spectrometric methods) have been described in numerous publications such as rapidity or flexibility. Furthermore, the method requires small amounts of sample at low solute concentration without the knowledge of its exact value. Potential impurities do not interfere with the calculation of pK_a, which is based on the migration time exclusively without peak quantification [11].

Usually, results are successfully compared to pK_a values obtained with two other reference methods as potentiometric or UV spectrophotometric techniques. The pK_a estimation could be achieved by a reversed-phase liquid chromatography method too [12].

Table 1
Electroosmotic mobility, μ_{eof}, as a function of electrolyte pH

pH	μ_{eof} ($\times 10^{-5}$ cm^2 V^{-1} s^{-1})
3.88	6.8
4.57	9.6
5.32	12.8
5.82	17.9
6.15	20.2
6.6	23.0
6.96	24.5
7.65	27.2
8.73	30.6
9.16	30.3

In this study, the electrophoretic behaviour of a series of imidazole derivatives has been investigated using CE with direct UV detection in background electrolyte adjusted at different pH values. Ionization constants have been determined and these values were confirmed by a UV spectrophotometric method and computer calculations.

2. Theoretical background

Imidazole derivatives **1**–**4**, **6** and **7** are basic compounds, mono-base B, where the acid–base dissociation constant K_a is defined as:

$$BH^+ \overset{K_a}{\rightleftarrows} B + H^+, \qquad K_a = \frac{[B](H^+)}{\gamma_{BH^+}[BH^+]} \tag{1}$$

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

In CE experiments, it is well established that pH influences the electrophoretic behaviour of studied substances. The electrophoretic mobility of the analyte (μ_e) is varied between a zero value (neutral form) to a maximum value (the ionized form, with $\mu_e = \mu_{BH^+}$). In the pH range around the pK_a of interest, the μ_e value is defined as follows:

$$\mu_e = (\%BH^+)\mu_{BH^+} \tag{2}$$

where %BH^+ is the molar fraction of the protonated imidazole derivative and μ_{BH^+} its electrophoretic mobility.

So, Eq. (2) becomes:

$$\mu_e = \frac{[BH^+]}{[B] + [BH^+]}\mu_{BH^+} \tag{3}$$

Finally, a relation between pH, pK_a and the electrophoretic mobility of compound can be written from Eqs. (1) and (3):

$$\mu_e = \frac{(H^+)}{(H^+) + \gamma_{BH^+} K_a} \mu_{BH^+} \quad (4)$$

The experimental μ_e value is calculated from:

$$\mu_e = \mu_{app} - \mu_{eof} = \frac{L_d L_t}{V} \left(\frac{1}{t_m} - \frac{1}{t_0} \right) \quad (5)$$

where μ_{app} is the apparent mobility of the solute, μ_{eof} the electroosmotic mobility of a neutral marker. μ_e, μ_{app}, μ_{eof} and μ_{BH^+} are expressed in $cm^2\,V^{-1}\,s^{-1}$. L_d (cm) is the distance from the injection point to the detector, L_t (cm) the total length of capillary and V (V) the applied voltage. t_m and t_0 (s) are the migration times of the analyte and the neutral marker (acetone), respectively.

Finally, Eq. (4) can be rearranged to give a linear expression between $1/\mu_e$ and $\gamma_{BH^+}/(\mu_{BH^+}(H^+))$, i.e.:

$$\frac{1}{\mu_e} = K_a \frac{\gamma_{BH^+}}{\mu_{BH^+}(H^+)} + \frac{1}{\mu_{BH^+}} \quad (6)$$

The K_a and the μ_{BH^+} values are directly obtained from the calculated slope and the ratio (1/intercept) with an activity coefficient, γ_{BH^+}, calculated from Debye–Hückel's theory at 25 °C according to Eq. (7):

$$\log \gamma = \frac{0.5085 Z^2 \sqrt{I}}{1 + 0.3281 \alpha \sqrt{I}} \quad (7)$$

$$I = \frac{1}{2} \sum C_i Z_i^2 \quad (8)$$

where α is the hydrated diameter of the ion, generally unknown but assumed to be 5 Å, C the molarity of the ion, Z the valency of the ion and I the ionic strength of the solution. In our experiments, γ_{BH^+} was considered as constant (calculated values are between 0.78 and 0.86 in the ionic strength range 0.023–0.074).

Eq. (4) is used for all studied mono-bases except for two compounds (**5** and **8**) which are described by two dissociation constants K_{a1} and K_{a2}. Compound **8** is a di-base B where $\mu_{BH_2^{2+}}$ and μ_{BH^+} are the electrophoretic mobilities of the first and the second protonated species formed from this analyte, respectively. μ_e is then defined as follows:

$$BH_2^{2+} \overset{K_{a1}}{\rightleftarrows} BH^+ + H^+$$
$$BH^+ \overset{K_{a2}}{\rightleftarrows} B + H^+$$

$$\mu_e = \frac{\gamma_{BH^+}(H^+)^2 \mu_{BH_2^{2+}} + \gamma_{BH_2^{2+}} K_{a1} (H^+) \mu_{BH^+}}{\gamma_{BH^+}(H^+)^2 + \gamma_{BH_2^{2+}} K_{a1} (H^+) + \gamma_{BH_2^{2+}} \gamma_{BH^+} K_{a1} K_{a2}} \quad (9)$$

where $\gamma_{BH_2^{2+}}$ and γ_{BH^+} are the activity coefficients of protonated species.

The ionization constant of compound **5**, named BA and characterised by amphoter property, was also determined using the method of Ishihama et al. [13]. μ_e is defined by:

$$BAH_2^+ \overset{K_{a1}}{\rightleftarrows} BAH + H^+$$
$$BAH \overset{K_{a2}}{\rightleftarrows} BA^- + H^+$$

$$\mu_e = \frac{\gamma_{BA^-}(H^+)^2 \mu_{BAH_2^+} + \gamma_{BAH_2^+} K_{a1} K_{a2} \mu_{BA^-}}{\gamma_{BA^-}(H^+)^2 + \gamma_{BAH_2^+} \gamma_{BA^-} K_{a1} (H^+) + \gamma_{BAH_2^+} K_{a1} K_{a1}} \quad (10)$$

where $\mu_{BAH_2^+}$, μ_{BA^-} and $\gamma_{BAH_2^+}$, γ_{BA^-} are the electrophoretic mobilities and the activity coefficients, respectively, of the corresponding charged species.

All pK_a values and the limiting mobilities of each individual ionized imidazole derivatives are then determined by adjusting the experimental values of these four-parameter equations through the utilisation of GraphPad Prism software.

3. Experimental

3.1. Reagents

New 3-methyl-6-[1-(imidazo-1-yl)-1-phenylmethyl] benzothiazolinone and 3-methyl-6-[1-(imidazo-1-yl)-1-phenylmethyl] benzoxazolinone **1**–**8** were synthesized by some of us. Phosphoric acid (d = 1.71, 85%), triethylamine (d = 0.72, 99%) and acetone 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 from a Milli-Q water purification system (Veolia Water, STI, Le Plessis Robinson, France).

3.2. Apparatus

Capillary electrophoretic experiments were performed using a Beckman P/ACE MDQ series capillary electrophoresis system with diode-array detector from 190 to 600 nm and system 32 Karat version 4.0 software (Beckman Instruments, Villepinte, France). The pH of the buffer solutions was measured using a combination pH electrode (Hanna Instruments, RI, USA).

All spectroscopy data were acquired on a Kontron UV-Vis spectrometer (Uvikon Instruments, Trappes, France). GraphPad Prism version 3.0 software (San Diego, CA, USA) was used to performed linear and non-linear regression analysis on the data. The computational predictions were made using ACD/pK_a software from Advanced Chemistry Development (Toronto, Canada).

3.3. Buffers preparation

Phosphate buffers of different pH values were prepared by addition of triethylamine to 25 mM H_3PO_4. The ionic strength of the buffers ranged from 0.023 to 0.074. For

practical reasons, it was pH′, the activity-corrected pH, not the thermodynamic pH, that was used to describe the acidity of the buffer.

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. Electrolyse was observed at both cathode and anode, with alkalisation and acidification of the buffer solutions, respectively. This drawback is prevented by the replacement of the buffer solutions every ten runs.

3.4. Samples preparation

For CE experiments, ethanol stock solutions of 2 g l^{-1} of all eight imidazole derivatives were prepared and used for further dilutions as necessary. The working solutions were done by diluting the stock solutions in each corresponding buffer. The final concentration was 100 mg l^{-1} in phosphate buffer with 1% acetone as a neutral marker. The solutions were always degassed in an ultrasonic bath prior to use.

Samples of 10 mg l^{-1} of a given compound were used for each spectroscopic determinations. The spectra of solutions were recorded in a wavelength range of 200–400 nm.

3.5. Electrophoretic conditions

The CE separations were all done with the conventional operating (anodic injection, mode short end). An uncoated fused-silica CE column (50.2 cm (40 cm to detector) × 50 μm i.d.) was obtained from Composite Metal Services (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 30 min, water for 30 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 psi; 1 psi = 6894.76 Pa). The capillary was operated at 20 kV with the current not exceeding 50 μA. The detection wavelengths were 194 and 218 nm for benzoxa- and benzothiazolinone, respectively. Six replicate injections were performed for each imidazole derivatives and average electrophoretic mobilities were used in the calculation of pK_a.

4. Results and discussion

4.1. Choice of electroosmotic marker

Acetone, acetonitrile, methanol, and dimethyl sulphoxide were tested as electroosmotic flow (EOF) marker substances. The best results (high absorbance and symmetrical peaks) were obtained by using acetone. In Table 1, the pH values and calculated μ_{eof} are given for a mixture of 1% acetone in 99% water. The relative standard deviation of the μ_{eof} values, calculated from three injections, was between 0.8% (neutral and alkaline conditions) and 3% (acidic region). The μ_{eof} first increases from pH 3.88 to 7.65 and then is stable in the range studied.

4.2. Choice of buffer pH range

To measure the pK_a values of the homogeneous chemical class as imidazole series described here, a phosphate buffer was chosen which permits to perform a wide pH range around the expected pK_a values. A pH range comprised between 3.88 and 9.16 has been used. The phosphate buffer at pH 3.88 allowed to measure the maximum electrophoretic mobility of the fully ionized imidazoles. To control the reproducibility of the μ_e, six injections for each mono-bases (compounds **1**–**4**, **6** and **7**) or for the di-base (compound **8**) were run from pH 3.88 to 9.16. The relative standard deviation of the effective mobilities was between 2.5% (neutral and alkaline conditions) and 1.1% (acidic region). For the ampholyte **5**, different relative standard deviation (from six runs) was obtained with 0.9% in alkaline or acidic conditions and 3% in neutral pH condition.

4.3. Choice of buffer concentrations

As previously described by Matoga et al. [9], 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 of 25 mM which induces a current intensity between 5.3 and 30.5 μA without Joule effect. Although different amounts of triethylamine were added to the buffer solutions to adjust pH, the activity coefficients of the overall buffer series remained almost constant (γ_{BH^+} calculated from 0.78 to 0.86 in the pH range).

4.4. Calculation of pK_a values

In the present paper, we studied the determination of the dissociation constant of imidazole derivatives by capillary zone electrophoresis as well as by spectrophotometric technique as comparison method. Since the buffers employed had different ionic strengths, electrophoretic mobilities were plotted against activity-corrected pH, of the buffer (pH′). Plots shown in Figs. 3 and 4 are obtained for the mono-base derivatives **1**–**4** and for the divalent compounds **5** and **8**, respectively. The pK_a can be graphically determined at the inflexion point of the sigmoidal curve. For precise determination of pK_a values, in the case of monovalent compound, we used the linear regression analysis on the data according to Eq. (6). An example of linear regression with imidazole derivative **2** is given in Fig. 5. As for all monovalent compounds, the correlation coefficient values, *r*, for the linear fit,

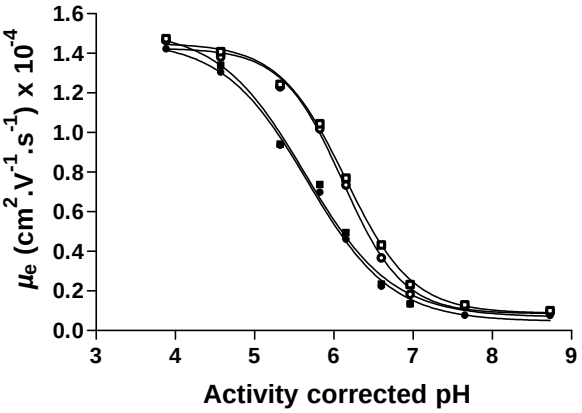

Fig. 3. Dependence of effective mobilities of four imidazole derivatives, mono-bases **1** (○), **2** (□), **3** (●) and **4** (■), vs. activity-corrected pH. Capillary: 50.2 cm (40 cm to detector) × 50 μm i.d.; field strength: 0.40 kV cm^{-1}; temperature: 25 °C; pressure injection: 5 s at 1 psi; detection: 194 and 218 nm for benzoxa- and benzothiazolinones.

was greater than 0.985. Slope and intercept values for compound **2** are $(5.88 \pm 0.31) \times 10^{-7}$ and $(6.73 \pm 0.85) \times 10^{-3}$, respectively. All p$K_a$ values calculated from the slopes (K_a) are given in Table 2.

As expected, we observed double inflexion point for the ampholyte **5**, whereas the curve observed for compound **8** shows a mono-sigmoid shape which gives a mean pK_a value of 5.80. This result could be explained by pK_a value of pyridine, with published value of 5.34 [14], close to the determined pK_a of imidazole groups in this series.

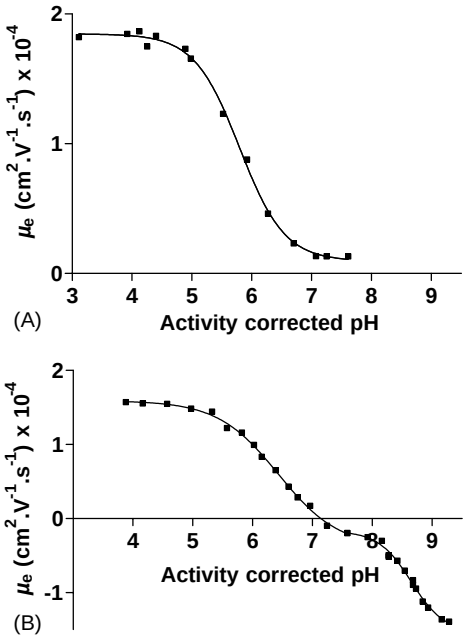

Fig. 4. Dependence of effective mobilities of imidazole derivatives vs. activity-corrected pH for: (A) ampholyte **5**; (B) di-base **8**. Capillary: 50.2 cm (40 cm to detector) × 50 μm i.d.; field strength: 0.40 kV cm^{-1}; temperature: 25 °C; pressure injection: 5 s at 1 psi; detection: 194 nm for benzoxazolinones.

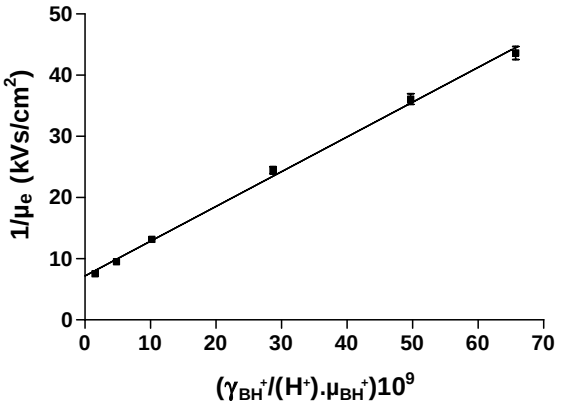

Fig. 5. Compound **2**. Plot of the reciprocal electrophoretic mobility (μ_e) as a function of $\gamma_{BH^+}/(\mu_{BH^+}(H^+))$ in a pH range of 4.42–7.76.

Table 2
pK_a values of the eight imidazole derivatives obtained by computer calculations, CE and UV spectroscopy

Compound	Computer calculations	CE	Spectroscopy
1	6.43	6.17 ± 0.05	6.20 ± 0.12
2	6.42	6.23 ± 0.05	6.25 ± 0.15
3	6.20	5.83 ± 0.07	5.87 ± 0.11
4	6.20	5.86 ± 0.06	5.89 ± 0.13
5	6.43	6.40 ± 0.07	–
	9.47	8.69 ± 0.04	–
6	–	6.05 ± 0.06	–
7	–	6.23 ± 0.03	–
8	–	5.80 ± 0.03[a]	–

[a] Mean value calculated from the mono-sigmoid curve.

The pK_a value of compound **5** can also be calculated by using a non-linear regression model on the experimental pair values of pH and μ_e as described in Section 5.

The pK_a values of the four imidazole derivatives **1**–**4** obtained by CE are close to those determined by UV spectroscopy, with values comprised between 5.80 and 6.23. An acceptable correlation is observed between CE and computer calculations (Table 2). 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 ionized functions, illustrated by results of compound **5**.

N-Substituted imidazole pK_a values have been reported for derivatives with methyl or phenyl substituents [15]. pK_a values were determined for imidazole and *N*-methylimidazole compounds with slight difference (pK_a = 7.43 and 7.20, respectively). On the other hand, aromatic substitutions should affect dramatically the basicity of the nitrogen illustrated by a pK_a value of 5.39 for the 4′-(imidazol-1-yl)acetophenone [15]. In the present study, significant differences of pK_a values are observed for *N*-substituted imidazoles with a *para*-cyanobenzyl (*p*-CN-benzyl) group compared to the analogues without an

electron-acceptor substituent placed on the aromatic ring. A direct comparison of the pK_a values of compounds **1** and **2** on one hand and compounds **3** and **4** on the other hand, demonstrated the basicity decrease of compounds with *para*-cyano substituent of aromatic ring in the benzoxa- or benzothiazolinone series. Replacement of the *p*-CN group by an weaker electron acceptor as *p*-Cl substituent on the aromatic ring (compound **3** versus compound **7**) leads to the pK_a value of compound **1** (p$K_a = 6.17 \pm 0.05$). Influence of the substituent position of the cyano group is investigated by comparison of the pK_a values of compounds **3** (*p*-CN, p$K_a = 5.83 \pm 0.07$) and **6** (*m*-CN, p$K_a = 6.05 \pm 0.06$). The *m*-CN group influence on the basicity of the endocyclic nitrogen is lower than the *p*-CN group. This result illustrated the expected opposites electronic effects of benzyl substituents tested, on the pK_a value of imidazole moiety.

In other way, no significant difference of pK_a values were observed between benzoxa- and benzothiazolinone series (compound **1** versus compound **2**, or compound **3** versus compound **4**) which demonstrated that the presence of O or S in the heterocycle did not induce significant change of imidazole basicity.

5. Conclusions

In this investigation, the determination of dissociation constants of eight *N*-substituted imidazole derivatives has been performed. The proposed CE method has been proven to be convenient to determine the pK_a values of these compounds with better measurement precisions than in spectrophotometric methodology. Moreover, CE allowed to work with very weak quantities of compounds (0.1 mg). Basic character of the *N*-substituted imidazole function is dependent of adjacent aromatic groups as observed by comparison of published values [15]. 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 CE and the spectroscopic methods as well as an acceptable correlation between the CE results and the data calculated using the ACD/pK_a software.

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. Furthermore, fluorimetric experiments have to be performed for affinity constant determinations of aromatase inhibitors with intrinsic fluorescent properties.

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