Measurement of acid dissociation constants and ionic mobilities of 3-nitro-tyrosine and 3-chloro-tyrosine by capillary zone electrophoresis

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The ionization constant (pKₐ) and limiting ionic mobility of 3-chlorotyrosine (CT) and 3-nitrotyrosine (NT) were determined in capillary zone electrophoresis (CZE) in a wide pH range. Measurements were carried out in a poly(ionic liquid) (PIL) modified capillary at a low pH(1.80–4.00) and a bare fused capillary at an upper pH (3.00–11.00). Electrostatic interaction between analytes and inner wall was suppressed dramatically. Furthermore, parameters usually empirically assumed were calculated from a simple theoretical model. Besides pKₐ and limiting ionic mobility, diameter of the hydrated ion was calculated as well. The former were inserted into the database of Peakmaster software, whose predictions led to satisfactory agreement with experimental runs.

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1. Introduction

3-Nitrotyrosine (NT) and chlorotyrosine (CT) (Fig. 1) were oxidation products of reactive oxidative species (ROS) and other radicals under inflammatory conditions. Chlorination of tyrosine decreases the ionization constant (pKₐ) of the phenolic group leading to altered protein conformation and function in the same way that nitration of tyrosine can alter enzymatic activity or protein function. Irreversible modification of functional tyrosine can seriously interfere with cellular regulation [1].

The pKₐ plays an important role in biochemical activity and in electrophoretic behavior along with other physicochemical parameters. Capillary zone electrophoresis (CZE) is a useful tool for determination of physicochemical parameters, e.g. pKₐ values and/or ionic mobilities. It has been applied for drugs [2,3], amino acids [4,5], peptides [6,7], phosphinic pseudopeptides [8], alkaloids [9], nucleotides [10] and organic acids [11]. Besides aqueous solution, pKₐ was measured in organic system [6,12] or aqueous-organic media [7,13]. Buffer system has been investigated to obtain a constant ionic strength and to avoid forming complexes or associates with analytes [14,15]. Meanwhile, modification of the inner surface has been studied, as it is time-consuming at low pH, where the electroosmotic flow (EOF) is too small to be detected and cations apt to be adsorbed onto the inner capillary wall. This can be solved by either suppression or acceleration of the EOF. The former applies modification with nonionic polymer either covalently (polyacrylamide [13,16]) or dynamically (polyvinyl alcohol (PVA) at very low pH [5]). The latter utilizes dynamic coating of a negative charged layer, Ceofix® [4,17], or a positively charged polymer, Micro-Coat [18], carboxymethyl chitosan [19] or polybrene (PB) [20]. For instance, Ceofix®, which can generate a large EOF even at pH as low as 1.5, has been developed for pKₐ measurements of acids, bases, ampholytes [17] and amino acids [4].

The electrostatic interaction can bring variations in pKₐ values [20]. Here, it is assumed that a cationic capillary, with reversed and detectable EOF, should be more suitable for cations at low pH due to electrostatic repulsion.

In previous studies in our lab [21,22], poly(1-vinyl-3-methylimidazolium bromide), a poly(ionic liquid) (PIL), was found rather stable to reverse the EOF and demonstrated excellent performances. In this study, PIL modified and fused capillaries were used to determine effective electrophoretic mobilities of CT and NT at low and high pH, respectively.

2. Theory

2.1. Determination of effective electrophoretic mobilities

The effective electrophoretic mobility μₑₑ of a target can be measured by its migration time (tₑₑ), time of the neutral marker...
where $\mu_{\text{lim}}$ is the limiting mobility in the CZE system, which is effective to solute up to a charge of $-4$ [23] and verified 0.1 M [5]

$$\mu = \mu_{\text{lim}} - |z| \left( B_1 z_+ \mu_{\text{lim}}^+ \frac{q}{1 + \sqrt{q}} + B_2 \right) \frac{\sqrt{T}}{1 + B_0 \sqrt{T}}$$

(8)

where $z_+$ and $z_-$ are charges of the cation and the anion, respectively; quantities $B_1$, $B_2$, and $B_3$ are functions of temperature and properties of the solvent; $a$ denotes diameter of the hydrated ion; $I$ is the ionic strength. For water as solvent at 298.15 K, $B_1 = 3.288$ nm$^{-1}$ molecule$^{-1/2}$ dm$^{2/3}$, $B_2 = 0.7853$ dm$^{3/2}$ molecule$^{-1/2}$, and $B_3 = 3.142 \times 10^{-9}$ mol dm$^{-3}$ V$^{-1}$ s$^{-1}$ dm$^{3/2}$ molecule$^{-1/2}$. The parameter $q$ can be obtained from limiting ion mobilities and charge numbers of ions:

$$q = \frac{z_+ |z_-|}{z_+ + |z_-|} \left( \frac{\mu_{\text{lim}}^+}{z_+ \mu_{\text{lim}}^+} + \frac{\mu_{\text{lim}}^-}{z_- \mu_{\text{lim}}^-} \right)$$

(9)

When Na$^+$ is the counter cation, its absolute cationic mobility is equal to $51.9 \times 10^{-9}$ m$^2$ V$^{-1}$ s$^{-1}$.

Diameter $a$ will be usually unknown. Typical values of small ions were from 0.1 to 1.1 nm [16]. In most cases, 0.5 nm is assumed, even for a peptide [24]. Obviously, this assumption is not much appropriate. In this study, diameter $a$ was obtained from the most simplified model,

$$\mu_{\text{lim}} = \frac{e z}{3 \pi \eta}$$

(10)

where $e$ represents the elementary charge $1.602 \times 10^{-19}$ C and $\eta$ is viscosity of the solvent, 0.8903 m N s$^{-2}$ for water as the solvent.

The activity coefficient $\gamma$ of an ion at a moderate ionic strength can be estimated from the extended Debye–Hückel law,

$$\log \gamma = -\frac{A |z| z_- \sqrt{T}}{1 + B_0 \sqrt{T}}$$

(11)

where parameter $A$ is related to solvent and temperature, for water at 298.15 K, $A = 0.5085$ mol$^{-1/2}$ dm$^{2/3}$.

The thermodynamic dissociation constant of the $i$th dissociation step, $pK_{ai}$, can be calculated,

$$pK_{ai} = pK'_{ai} - \log \gamma_{Hn^-X^i-} + \log \gamma_{Hn^-iX^i-1}$$

(12)

### 3. Materials and methods

#### 3.1. Standards and reagents

3-Nitrotyrosine (NT, 98%) and 3-chlorotyrosine (CT, 97%) were purchased from Alfa Aesar (Tianjin, China), 3-(Cyclohexylamino)-1-propanesulfonic acid (CAPS) and N-(2-Hydroxethyl)piperazine-N’-(2-ethanesulfonic acid) (HEPES) were obtained from Sangon (Shanghai, China). Sodium hydroxide (96%) was supplied by Rionlon Bohua (Tianjin) Pharmaceutical & Chemical Co., Ltd. (Tianjin, China). Glycine was from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Acetic acid (glacial), formic acid and DMSO were from Tianjin Chemical Reagent Co., Ltd. (Tianjin, China). Phosphoric acid (85%) was obtained from Liangyou Chemical Reagent (Beijing, China). All reagents were of A.R. grade or higher. P.I.L. was synthesized previously in our lab [21,22].

#### 3.2. CE instrumentation

Experiments were performed on an Agilent CE system (Agilent Technologies, Beijing, China) equipped with a UV DAD utilizing ChemStation (Rev.A 09.03). Detection was monitored at 200, 214 and 232 nm.

Fused silica capillaries (Yongnian Country Reafine Chromatography, Hebei, China) of 48.5 cm (effective length 40 cm) × 50 μm i.d.
Table 1
The set of BGEs at constant ionic strength (I = 25 mM).

<table>
<thead>
<tr>
<th>Buffering component (mM)</th>
<th>pK₀</th>
<th>pH range</th>
<th>Counter cation (mM)</th>
<th>Current (µA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAPS</td>
<td>10.4</td>
<td>10.2–11.0</td>
<td>Na (25)</td>
<td>16</td>
</tr>
<tr>
<td>Glycine</td>
<td>9.7</td>
<td>8.6–10.6</td>
<td>Na (25)</td>
<td>18</td>
</tr>
<tr>
<td>HEPES</td>
<td>7.5</td>
<td>6.6–8.6</td>
<td>Na (25)</td>
<td>17</td>
</tr>
<tr>
<td>Acetate</td>
<td>4.76</td>
<td>3.8–5.6</td>
<td>Na (25) ± 1</td>
<td>±19</td>
</tr>
<tr>
<td>Formate</td>
<td>3.71</td>
<td>2.8–4.0</td>
<td>Na (25)</td>
<td>-20</td>
</tr>
<tr>
<td>Phosphate (28)</td>
<td>2.16</td>
<td>2.94</td>
<td>Na (24)</td>
<td>-20</td>
</tr>
<tr>
<td>Phosphate (31)</td>
<td>2.16</td>
<td>2.77</td>
<td>Na (24) − 14</td>
<td></td>
</tr>
<tr>
<td>Phosphate (34)</td>
<td>2.16</td>
<td>2.58</td>
<td>Na (23)</td>
<td>-16</td>
</tr>
<tr>
<td>Phosphate (36.5)</td>
<td>2.16</td>
<td>2.35</td>
<td>Na (20)</td>
<td>-17</td>
</tr>
<tr>
<td>Phosphate (45)</td>
<td>2.16</td>
<td>2.18</td>
<td>Na (18)</td>
<td>-22</td>
</tr>
<tr>
<td>Phosphate (55)</td>
<td>2.16</td>
<td>2.00</td>
<td>Na (15)</td>
<td>-20</td>
</tr>
<tr>
<td>Phosphate (75)</td>
<td>2.16</td>
<td>1.75</td>
<td>Na (7)</td>
<td>-27</td>
</tr>
</tbody>
</table>

Superscripts a and b denote – 12 and – 8 kV, respectively.

Table 2
Results from nonlinear fit.

<table>
<thead>
<tr>
<th></th>
<th>pK₀ ± SD</th>
<th>μ ± SD (× 10⁻⁹ m² s⁻¹ V⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(+1)</td>
<td>(−1)</td>
</tr>
<tr>
<td></td>
<td>(+1)</td>
<td>(−1)</td>
</tr>
<tr>
<td>CT</td>
<td>0.9993</td>
<td>2.19 ± 0.02</td>
</tr>
<tr>
<td>NT</td>
<td>0.9996</td>
<td>2.06 ± 0.01</td>
</tr>
</tbody>
</table>

were used for separation. Before use, the new capillary was flushed (≈940 mbar) with methanol (10 min), 1 M HCl (20 min), 1 M NaOH (20 min). For PIL capillary, it was followed by 10 min 1 g L⁻¹ PIL. Capillaries were then rinsed with BGE (5 min). Between runs, the bared capillary was flushed with 0.1 M NaOH (2 min) and the BGE (3 min); while in the PIL capillary, 1 g L⁻¹ PIL was used instead of 0.1 M NaOH.

The capillary cartridge was thermostated 25 °C. Electrophoresis was carried out at 20 kV, except in phosphate buffer. The buffer pH was measured using a Sartorius PB-10 pH meter (Beijing, China) at 25 °C.

3.3. Samples and BGEs

Stock solutions of 1 mM NT were dissolved in 5 mM NaOH for being slightly soluble in water, while 1 mM CT in 2 mM HCl to avoid hydrolysis.

Working samples were prepared in a concentration range of 0.25–0.05 mM by diluting the stock solutions with BGE, containing 0.1% (v/v) DMSO as an EOF marker. Either an individual or a mixed sample was injected at 30 mbar, 3 s.

The BGEs were prepared with ionic strength 25 mM over the pH range 1.80–11.00. Peakmaster 5.2 by Gas [25] helps to predict the ionic strength, buffer capacity and conductivity of different buffer system. The increment of pH units was set at 0.2–1.0. Compositions of buffer system are listed in Table 1. Constituents of phosphate buffers at theoretical pH values of 3.0, 2.8, 2.6, 2.4, 2.2, 2.0 and 1.8 were calculated, and measured pH of the corresponding buffer was shown in Table 1.

Besides, 50 mM phosphate (3.5 mM NaH₂PO₄ and 46.5 mM Na₂HPO₄, pH 8.00) was used to test data obtained from this study. Samples and electrolytes were filtered through 0.45 µm filters (Tengda, Tianjin, China) before use.

4. Results and discussion

4.1. Selection of experimental conditions

BGEs covering a pH range of 1.80–11.00 were selected, shown in Table 1. Univalent weak acid is easy to prepare a buffer of a constant ionic strength, with which no or minimum associates or complex anion could form [15]. A counter ion Na⁺ was used in a constant concentration to keep the ionic strength. The ionic strength was at 25 mM and the pH range of a certain buffer system was pK₀ ± 1, in order to obtain low Joule heating and sufficient buffer capacity.

The power was no more than 0.3 W to avoid Joule heating. The applied voltage was reduced for phosphate buffers, whose conductivities were much higher than those of the others.

4.2. CZE separation and detection in a PIL coated capillary and a bare capillary

A cationic polymer PIL was used to reverse the EOF and to achieve fast and efficient separation in the pH range 1.80–4.00. The EOFs generated were ranging from −55 × 10⁻⁹ to −34 × 10⁻⁹ m² s⁻¹ V⁻¹ in phosphate buffers, which enabled detection of the analytes in negative polarity. Peak efficiencies were in

Table 3
Absolute ionic mobilities and pK₀ values adjusted with ionic strength.

<table>
<thead>
<tr>
<th>Analytes (charge)</th>
<th>q</th>
<th>a (nm)</th>
<th>Limiting mobility (× 10⁻⁹ m² s⁻¹ V⁻¹)</th>
<th>pK₀</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT (+1)</td>
<td>0.5077</td>
<td>0.9656</td>
<td>23.67</td>
<td>2.14</td>
<td>2.02a</td>
</tr>
<tr>
<td>(-1)</td>
<td>0.5146</td>
<td>1.0592</td>
<td>21.76</td>
<td>8.01</td>
<td>8.0b, 8.3h</td>
</tr>
<tr>
<td>(-2)</td>
<td>0.4560</td>
<td>1.0554</td>
<td>-44.55</td>
<td>9.75</td>
<td>9.34a</td>
</tr>
<tr>
<td>NT (+1)</td>
<td>0.5024</td>
<td>0.9008</td>
<td>25.21</td>
<td>2.01</td>
<td>2.20a</td>
</tr>
<tr>
<td>(-1)</td>
<td>0.5030</td>
<td>0.9078</td>
<td>-25.04</td>
<td>6.87</td>
<td>7.20b, 6.74, 6.94f</td>
</tr>
<tr>
<td>(-2)</td>
<td>0.4539</td>
<td>1.0244</td>
<td>-45.78</td>
<td>9.59</td>
<td>9.11a</td>
</tr>
</tbody>
</table>

Superscripts a, b and c denote Refs. [26], [27] and [28], respectively; d was the pK₀ value calculated from Ref. [29].
the range of 136,000–224,000 plates m$^{-1}$. Adsorption was greatly eliminated due to electrostatic repulsion.

Analytes were determined in a bare fused capillary at pH ranging from 3.00 to 11.00. Effective separations can be observed at pH higher than 6.60, where a large EOF was produced in a range from $58 \times 10^{-9}$ to $66 \times 10^{-9}$ m$^2$ s$^{-1}$ V$^{-1}$ and analytes were in an anionic form. Thus the interaction between the capillary inner surfaces with analytes was reduced as well.

### 4.3. Ionization constants and limiting ionic mobilities

Fig. 2 shows the effective electrophoretic mobility as a function of pH. Eq. (7) was used for nonlinear curve fit. The regression result was shown in Table 2. Obviously, the data points adhered very well to the fitting curve. The relative standards were less than 0.02 and 0.50 for $pK_a$ values and actual ionic mobilities, respectively.

The $pK_a$ values and limiting ionic mobilities calculated with Eqs. (8)–(12) were listed in Table 3, together with reference data from literature. Parameters $p$ and $q$ were given as well. For univalent compounds, $q$ values were in the range of 0.502–0.515; while in case $z = -2$, $q$ values were ranging from 0.454 to 0.456. The hydrodynamic diameters of ions were in the range of 0.90–1.06 nm, with corresponding $Ba$ values 2.9–3.5. Obviously, those usually assumed $Ba$ values 1.5 [5], 1.65 [11] or 2.4 [23] are not appropriate to these ions. Therefore, our calculation should be superior to those assumptions. Generally, the ion size is dependent on conformation and hydration number. The small size of CT (−2) is referred to decreased hydration number as a result of deprotonation of −NH$_3^+$ group. Exceptionally, diameter of NT (−1) is smaller than that of NT (−1), and close to that of NT (+1). It is inferred that there exists an interaction between the −NH$_3^+$ group and the −NO$_2$ group of NT (−1), resulting in a compact conformation. Deprotonation of −NH$_3^+$ group will make the conformation extended. Along with Eq. (10), the differences of limiting ionic mobilities can be explained. The $pK_a$ values obtained in this study were very close to those reported within a few hundredths variation. These data in our study are in a reasonable range, as they are in the range 1.82–2.83 and 8.80–10.60 for −COOH and −NH$_3^+$ groups in amino acids, respectively [30]. Further comparison was not available in view of lack of adequate reference data to our knowledge.

**Fig. 2.** Relationship between pH and effective electrophoretic mobilities of NT (empty) and CT (black).

**Fig. 3.** Experimental (top) and simulated (bottom) electropherograms. 1, NT; 2, CT. BGE: phosphate, pH 2.00 (A and B); glycine, pH 8.20 (C and D); 50 mM phosphate, pH 8.00 (E and F). Voltage: 8 kV (A and B); 10 kV (E and F); 20 kV (C and D). Capillary: P1L (A and B); bare-fused silica (C–F). Other conditions were as described in Sections 3.2 and 3.3.
In order to determine the reliability of these data, simulations were compared with experimental results (Fig. 3). The Limiting ionic mobilities and $pK_a$ values from Table 3 were added into the database of PeakMaster 5.2. Simulations agreed with experimental runs both in the PIL capillary (e.g. Fig. 3A and B) and in the bare one (e.g. Fig. 3C and D). Besides, an excellent agreement was obtained in 50 mM phosphate buffer at pH 8.0 (Fig. 3E and F), whose ionic strength was up to 143 mM. The relative deviations between simulation and corresponding experiment were within 1.8%. Therefore, these data are credible and can be further used for predictions of either separation or concentration if the ion interactions are ignored. Meanwhile, determination of cations in the PIL capillary and anions in the fused silica capillary was a successful trial.

5. Conclusions

Great care should be taken to determine limiting ionic mobilities and $pK_a$ values in CZE. Attention should be paid to not only experimental conditions but also parameters used in calculations. We suggest that charge of both the analyte and capillary inner surface be taken into consideration for determination of $pK_a$ and ionic mobility, and electrostatic interaction be avoided. If diameter of a compound is unknown, formulas in this paper will be helpful.

Acknowledgments

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References