A simple capillary electrophoresis with electrochemical detection method for determination of the hydrolysis rate constant of chlorogenic acid

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A method based on the kinetics stability study on hydrolysis of chlorogenic acid by capillary zone electrophoresis with electrochemical detection (CE-ED) has been developed in this paper. Both cyclic and hydrodynamic voltammograms of chlorogenic acid and its hydrolysis product caffeic acid have been investigated. The conditions for separation of chlorogenic acid and caffeic acid, such as the buffer pH and concentration, the separation voltage, and the injection time have been optimized. Under the optimum CE running conditions, the effects of reaction temperature and pH values of the hydrolysis solutions on the hydrolysis rate constants were further studied. The hydrolysis rate constants of chlorogenic acid were obtained from the concentration change of hydrolysis during the process of hydrolysis. Based on the fact, a simple and economical method for the determination of the hydrolysis rate constant and activation energy of hydrolysis reaction has been developed.

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own limitation in the aspect of sensitivity. In addition, for UV spectrophotometer, complicated pretreatments are usually required. Capillary electrophoresis (CE) as an important separation technology with the advantage of minimal sample volume requirement, short analysis time and high separation efficiency, it has been used as a powerful tool for the drug analysis, such as determination of the main component, estimation of impurities and the separation of the chiral substance [9–13]. More recently, CE has been developed to measure the physicochemical constants and the kinetic study of some reactions [6,14–16]. But there are seldom studies focusing on the hydrolysis rate constant of compound by using CE [17–21].

CE coupling with electrochemical detection (CE-ED) offers high sensitivity and good selectivity for separation and detection of electroactive analytes, and it has been widely applied in the quantitative analysis including the main active ingredients of crude drugs [22–24]. There were some reports about quantitative determinations of chlorogenic acid and caffeic acid in crude drugs [25–30], but still no description is related to the kinetic study for hydrolysis of chlorogenic acid. In this study, CE-ED was used as an effective method for directly measuring the hydrolysis rate constant and activation energy of chlorogenic acid, and demonstrating its use for studying the hydrolysis reaction. Under the optimum separation conditions, chlorogenic acid and its hydrolysate caffeic acid could be fast separated within 7 min, and the experimental results indicated that this method was easy, efficient and intuitive to obtain the hydrolysis rate constant and activation energy.

2. Theory

As mentioned in introduction, chlorogenic acid can be hydrolyzed to produce caffeic acid and quinic acid in alkalescent aqueous solution. The hydrolysis reaction can be described as Fig. 1.

As water is superfluous during the hydrolysis, the concentration of water can be considered to be constant in the reaction. So, the hydrolysis of chlorogenic acid is a first-order reaction and the kinetic equation is described as follow:

\[
\frac{dC}{dt} = kC
\]

The kinetics equation can be also expressed as,

\[
\ln C = -kt + \ln C_0
\]

where \( C \) is the concentration of chlorogenic acid at the hydrolysis time \( t \), which can be determined by CE-ED, and \( C_0 \) is the initial concentration of chlorogenic acid, then \( k \) is the rate constant of hydrolysis reaction. Since the kinetic reaction is first-order, when \( \ln C \) is plotted against \( t \), a straight line can be obtained, and the slope of the line is the first-order rate constant \( k \).

As shown in Arrhenius equation, the relationship between \( k \) and absolute temperature of the reaction \( T \) can be described as follow:

\[
\ln k = A e^{-E/(RT)}
\]

where \( A \) is apparent frequency factor, \( E \) is activation energy of the reaction; \( R \) is the mole gas constant (8.314 J K\(^{-1}\) mol\(^{-1}\)). In addition, Eq. (3) can also be expressed as,

\[
\ln k = -\frac{E}{RT} + \ln A
\]

when \( \ln k \) is plotted with \( T^{-1} \), a straight line (slope = \(-E/R\)) is obtained. So the activation energy \( E \) can be calculated according to the slope (\( E = -\text{slope} \times R \)).

3. Experimental

3.1. Materials

Chlorogenic acid and caffeic acid were purchased from the Chinese Institute of Biological Products Control (Beijing, China). Other chemicals were of analytical-reagent grade. All solutions were freshly prepared with doubly distilled water and filtrated through a 0.22 \( \mu \)m membrane filter before use.

Stock solution of chlorogenic acid (1.8 mmol/L) and caffeic acid (3.4 mmol/L) were prepared by using 5% (v/v) methanol–buffer solution (pH 7.0, 8 mmol/L KH\(_2\)PO\(_4\)–4 mmol/L Na\(_2\)B\(_4\)O\(_7\) solution) and diluted to the desired concentration with the running buffer just prior to use. Both solutions were kept in a 4 °C refrigerator and were stable for at least 2 months.

Buffer solution was prepared from the mixture of 0.1 mol/L KH\(_2\)PO\(_4\) and 0.05 mol/L Na\(_2\)B\(_4\)O\(_7\). And then the desired concentrations of buffers were obtained by diluting with distilled water. The pH values of buffers were adjusted accurately by 0.1 mol/L H\(_2\)PO\(_4\) or 0.1 mol/L KOH with a pH meter. The running buffer used for electrophoresis was 8 mmol/L KH\(_2\)PO\(_4\)–4 mmol/L Na\(_2\)B\(_4\)O\(_7\), unless indicated otherwise.

3.2. Apparatus

The CE-ED assembly was self-constructed in this laboratory and was similar to that described previously [31]. A high-voltage power supply (Shanghai Institute of Nuclear Research, China) provided a voltage of up to 30 kV between the ends of the capillary. The inlet end of the capillary was held at a positive potential and the outlet end was grounded; the apparatus was housed in an interlock box to prevent the operator from accidental shock. It was reported that for end-column amperometric detection method when the capillary internal diameters was less than 25 \( \mu \)m, the potential field effect on background noise could be negligible [32,33]. In this experiment, a 30 cm length of 25 \( \mu \)m i.d., 360 o.d. \( \mu \)m uncoated fused-silica capillary was used (Yongnian Optical Fiber Factory, Heibe, China). The unused capillary had been flushed with 0.1 mol/L sodium hydroxide solution for four hours before use, then rinsed with 0.1 mol/L HCl and doubly distilled water for 10 min each. Between run, the capillary was rinsed with 0.05 mol/L sodium hydroxide solution, doubly distilled water and running buffer for 5 min, respectively.

A conventional three-electrode electrochemical cell consisting of a 300 \( \mu \)m diameter carbon disc working electrode, a platinum auxiliary electrode, and a Ag/AgCl (saturated KCl) electrode as reference electrode, was connected to a BAS LC-4C electrochemical detector (Bioanalytical Systems Inc., West Lafayette, IN, USA). Before use, the working electrode was successively polished with emery paper and alumina powder, sonicated in water, and finally exactly leveled with the outlet of the capillary and shaped a wall-jet configuration. The data were recorded by TL9902 analytical system of chromatogram.

The hydrolysis was carried out in a constant temperature water bath (Medical Treatment Instrument Factory, Jiangsu, China).

3.3. Hydrolysis procedures

The constant temperature water bath was adjusted to the desired temperatures at which the hydrolysis was carried out (70, 80 and 90 °C, respectively). One tube was filled with 600 \( \mu \)L of 0.12 mmol/L chlorogenic acid solution and the other was full of the same volume of 8 mmol/L KH\(_2\)PO\(_4\)–4 mmol/L Na\(_2\)B\(_4\)O\(_7\) buffer solutions. Both of them were placed into the constant temperature water bath for 20 min. The buffer solution was then added to the above tube containing chlorogenic acid and mixed by shaking. In a
certain interval time, an accurate volume of 100 μL of hydrolysates was transferred to another tube, and then ice was used in order to terminate the hydrolysis reaction. At last the solution was analyzed by CE-ED, and the measurement was repeated for three times at least. The hydrolysis times ranged from 1 to 8 h.

4. Results and discussion

4.1. Electrochemical characteristic

Due to the fact that the molecular structure of both chlorogenic acid and caffeic acid possessing hydroxyl groups, the two analytes are anticipated to be electroactive at electrode, and this has been verified by cyclic voltammetry. The optimal detection potential for CE was determined by measuring the hydrodynamic voltammograms (HDVs) potential ranging from 800 to 1050 mV for chlorogenic acid and caffeic acid. As a compromise of higher current responses and lower noise, the applied potential of working electrode was maintained at ±900 mV (versus Ag/AgCl). The experiment indicated that the working electrodes could present good stability and high reproducibility at the optimum potential for more than two months.

4.2. Optimum conditions for electrophoresis

To determine the optimum conditions for the separation and detection of chlorogenic acid and caffeic acid, a standard mixture solution was analyzed to optimize the composition of the buffers and the voltage.

In this study, single constituent buffer was firstly considered, but poor Rs were obtained. It was found that the satisfied separation and good sensitivity could be obtained when the KH₂PO₄–Na₂B₄O₇ buffer solution was used as the carrier buffer. This is likely due to the following reasons. On the one hand, as demonstrated in the literature [34], B₄O₇²⁻ and hydroxyl of glucose in the structure of chlorogenic acid could form ligand, then Rs of chlorogenic acid and caffeic acid would be improved. On the other hand, it was also described [35] that the effective mobility (μₑ) was increased with the cation in buffer solution according to the order of Li, Na, and K. As a result, it was considered that KH₂PO₄ was more helpful than NaH₂PO₄ for this analysis system.

Both pH and concentration of the buffer play a key role in CE method. It had been mentioned that chlorogenic acid could be hydrolyzed to produce caffeic acid and quinic acid in alkalescent aqueous solution, so neutral solution was suitable for the running buffer of electrophoresis. In this study, buffer solutions with different pH values (6.8, 7.0, 7.2, 7.4 and 7.6) were tested. In the range from 7.0 to 7.6, the two current response peaks were separated completely, however, their current response reduced with the increase of the pH value. So, pH 7.0 was selected as the optimum pH. The effect of the concentration of the buffer was also investigated. On the promise of enough resolution of two analytes, low concentration of buffer was employed to reduce the migration time and improve sensitivity. As a compromise, 8 mmol/L KH₂PO₄–4 mmol/L Na₂B₄O₇ solution was selected.

The effect of separation voltage on separation efficiency of CZE was investigated in the range of 12–24 kV. In this range, the migration times of the analytes were significantly shortened and the current signals were increased a little when the separation voltage increased. However, when it was higher than 18 kV, the Rs were reduced to less than 1.6. Therefore, 18 kV was selected as the separation voltage. Electrokinetic sampling was used in our experiment. The injection time ranging from 4 to 14 s was optimized, and 10 s was selected as the injection time in this experiment.

The typical electropherogram of a standard mixture solution consisted of chlorogenic acid (8.3 × 10⁻⁵ mol/L) and caffeic acid (1.5 × 10⁻⁴ mol/L) was obtained under the optimum conditions and shown in Fig. 2. It can be seen from Fig. 2 that these two analytes were completely separated within 7 min at room temperature.

4.3. Validity of CE-AD method

Under the optimized conditions, a standard mixture solution was tested to determine the repeatability of the peak current and migration time for the two analytes. The relative standard deviations (RSDs) of peak current and migration time were 2.7% and 1.1% for chlorogenic acid, 3.4% and 1.1% for caffeic acid, respectively (n = 7).

![Fig. 2. Electrophorograms of standard mixture.](image-url)
A series of the standard mixture solutions of chlorogenic acid and caffeic acid with the concentration in the range of 0.48–122 and 0.76–226 μmol/L, respectively, were tested to determine the linearity for this pair of hydro-analytes at the carbon disc electrode (n = 3). The detection limits obtained was 0.09 and 0.19 μmol/L, respectively by IUPAC recommendation (LOD = 3σ/b). The results of detection limits and regression analysis on calibration curves are presented in Table 1. The calibration curves exhibited an excellent linear response, R = 0.9995 and 0.9991 for chlorogenic acid and caffeic acid, respectively over three orders magnitude of the concentration.

Furthermore, the recoveries of spiked sample was determined to examine the accuracy of the proposed method. A standard mixture solution of 5.6 × 10⁻⁵ and 1.1 × 10⁻⁴ mol/L of chlorogenic acid and caffeic acid, respectively, was selected as the spiked sample. Then two different concentrations of standard mixture solution were added to the spiked sample, and the recoveries of chlorogenic acid and caffeic acid were in the range of 105–111% and 98.1–116%, respectively.

### 4.4. Kinetic study on hydrolysis of chlorogenic acid

According to the Section 3.3, the hydrolysis of chlorogenic acid was carried out in the constant temperature water bath (90 °C). Fig. 3 shows the typical electropherograms of chlorogenic acid and hydrolyzate caffeic acid at 90 °C in 8 mmol/L KH₂PO₄–4 mmol/L Na₂B₄O₇ (pH 9.0) buffer solution when the hydrolysis time were 1, 2, 3, and 4 h, respectively. As shown in Fig. 3, the change of the peak currents of peak 1 and peak 2 demonstrates the change of concentrations of chlorogenic acid and caffeic acid during the process of hydrolysis. The peak current of chlorogenic acid was decreased gradually with the hydrolysis time increased, while the peak current of caffeic acid was increased at the same time.

As we known, kinetic constant of hydrolysis depends on the temperature closely. The hydrolysis of chlorogenic acid at temperature of 70 and 80 °C was carried out in the same way and then the variation of the concentration with hydrolysis time was observed. Fig. 4 shows the relationship between lnC and t for chlorogenic acid hydrolysis reaction (at pH 9.0) at various temperatures (at 70, 80, 90 °C), and it exhibits a good linearity. The experimental results indicated that the hydrolysis velocity decreased with the decreasing of reaction temperature under the same acidity (pH 9.0).

Then the hydrolytic reaction in a buffer solutions with pH of 8.0 was carried out, and the result showed that it would take at least 15 h to complete the hydrolysis reaction at 90 °C under pH 8.0, while only 12 h under pH 9.0. This indicated that the hydrolysis rate constant was closely related to the alkalinity of the hydrolysis solution. Higher alkalinity would be helpful to increase the reaction rate.

In the light of Eq. (2) of Section 2, a straight line can be obtained by plotting lnC against t (h) at different alkalinity of the hydrolysis solution. The slope of the line is −k, and k is the rate constant of hydrolysis reaction. In order to obtain the activation energy of chlorogenic acid hydrolysis reaction, the plot of lnk versus 1/T is mapping according to Eq. (4) of Section 2. A good linearity between lnk and absolute temperature T⁻¹ is approximately in accordance with Van’t Hoff Rule. The regression equation, correlation coefficient, and the calculated activation energy in two pH values solutions are shown in Table 2. The activation energies under pH

### Table 1
Regression equations and the detection limits.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Regression equation (Y = a + bX)</th>
<th>Correlation coefficient</th>
<th>Linear range (μmol/L)</th>
<th>Detection limits (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorogenic</td>
<td>Y = −0.1906 + 0.1071X</td>
<td>0.9995</td>
<td>0.48–122</td>
<td>0.09</td>
</tr>
<tr>
<td>Caffeic</td>
<td>Y = −0.2102 + 0.0559X</td>
<td>0.9991</td>
<td>0.76–226</td>
<td>0.19</td>
</tr>
</tbody>
</table>

a Conditions were the same as in Fig. 2.

b Where Y is the peak current (in 1 nA) and X is the compound concentration (in μmol/L).

c The detection limits were calculated according to IUPAC recommendations (LOD = 3σ/b).

![Fig. 3](image)  
**Fig. 3.** Electrophorogram showing of the hydrolysis of chlorogenic acid. Conditions were the same as in Fig. 2.

![Fig. 4](image)  
**Fig. 4.** Relationship between lnC and t for chlorogenic acid hydrolysis reaction at various temperatures in pH 9.0 solution.

### Table 2
Influence of pH on activation energy of chlorogenic acid hydrolysis.

| pH   | Regression equation | Correlation coefficient | E_a (kJ mol⁻¹) |
|------|---------------------|-------------------------|______________|
| 8.0  | ln k = −8615.0/T + 22.842 | 0.9986                  | 71.62        |
| 9.0  | ln k = −7007.7/T + 18.436 | 0.9994                  | 58.26        |

a Conditions were the same as in Fig. 2.
8.0 and pH 9.0 are 71.62 and 58.26 kJ mol$^{-1}$, respectively. It was shown that higher pH would lower the activation energy of hydrolysis reaction. The good straight-line illustrated $E$ was approximately a constant, which is independent of $T$ in the tested temperature range.

5. Conclusion

In this work, capillary electrophoresis with electrochemical detection was found to be a useful technique for studying the kinetic aspects of chlorogenic acid hydrolysis in aqueous solution. Except for the determination of hydrolysis rate constant, the method would promise to be applied to the study of other physico-chemical constant, such as the dissociation constant ($pK_a$), binding constant and so on for other compounds existed in the crude drugs.

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