Separation and Analysis of Chlorpromazine and Demethylchlorpromazine by Capillary Electrophoresis Coupled with Electrochemiluminescence

Fuxiu Yang¹, Zhaokuai Peng¹,², Chunxiu Gu¹,², Baining Liu¹,², Kaowen Zhou¹,²,*

¹Biochemical Engineering College, Beijing Union University, Beijing 100023, China
²Beijing Key Laboratory of Biomass Waste Resource Utilization, Beijing 100023, China
*E-mail: zhoukaowen@buu.edu.cn

Received: 4 February 2019 / Accepted: 24 March 2019 / Published: 10 June 2019

Demethylchlorpromazine (DMCPZ) is one of the main metabolites of chlorpromazine (CPZ). DMCPZ has only one methyl less than CPZ. The structural difference between them is too small to separate them by conventional capillary electrophoresis (CE). However, proper addition of additives in the separation buffer can improve their separation performance. The parameters related to CE separation were carefully studied. The optimized CE conditions were separation buffer of 17.50% iso-propyl alcohol aqueous solution (v/v) containing 21mmol/L phosphate (pH 5.3), separation voltage of 13.1 kV, sample injection time of 6 s and sample injection voltage of 11.5 kV. Combined with electrochemiluminescence (CEL) detection technology, a separation and analysis method of CPZ and DMCPZ by improved CE coupled with end-column CEL was established. The limits of detection (3σ) of this method were 8.1×10⁻⁷ mg/mL for CPZ and 8.9×10⁻⁶ mg/mL for DMCPZ. The relative standard deviations were less than 2.5% for ECL intensity and less than 1.2% for migration time. This method had many advantages, such as fast speed, high sensitivity, small sample injection, and free from interference, and was successfully applied to simultaneously determine CPZ and DMCPZ in whole urine sample of pet dog.

Keywords: Improved capillary electrophoresis; Electrochemiluminescence; Chlorpromazine; Demethylchlorpromazine; Urine sample

1. INTRODUCTION

Tris (2,2’-bipyridyl) ruthenium (II) (Ru(bpy)₃²⁺)-based electrochemiluminescence (ECL) for detecting organic amine compounds has attracted much attention due to its inherent high sensitivity and selectivity in the past decades. Capillary electrophoresis (CE) is an efficient method for separation of biochemical and medical analytes because of its strong separation ability, short analysis time and less sample consumption. CE separation with end-column ECL detection (CE-ECL) has been
extensively studied and utilized for the analysis of various drugs [1–21], antibiotics [22–24], enzymes [25], alkaloids [26–29], amines [30–34], hormones [35] and pesticide residues [36,37] in different foods, pharmaceuticals, animals and plants.

Chlorpromazine (CPZ), a representative drug of phenothiazine, is an antagonist of the central dopamine receptor, and has many pharmacological activities [38]. In vivo, CPZ can be bio-transformed into a variety of metabolites which most belong to the phenothiazine category, such as chlorpromazine sulfoxide (CPZSO), 2-chlorophenothiazine (2-CPTZ), demethylchlorpromazine (DMCPZ) and so on. DMCPZ has only one methyl less than CPZ (see Figure 1). The structural difference between them is too small to separate them by conventional CE in our previous work [17]. Simultaneous determination of CPZ and its metabolites in urine, however, is extremely important to realize the economic effect and pharmacokinetics of CPZ.

In this work, firstly, an improved CE method for the separation of CPZ and DMCPZ was proposed, and the separation conditions were optimized. Combined with CEL detection technology, then, a rapid separation and analysis method of CPZ and DMCPZ in whole urine sample by improved CE coupled with end-column CEL was established and validated. The results showed that the present method is sensitive and reliable for the simultaneous determination of CPZ and DMCPZ, which is helpful for the study of its effectiveness and safety evaluation, rational dose design and the underlying mechanisms.

![Figure 1. Structure of chlorpromazine and demethylchlorpromazine.](image)

### 2. EXPERIMENTAL

#### 2.1. Chemicals

Tris(2,2’-bipyridyl)ruthenium(II) dichloride hexahydrate (Ru(bpy)3Cl2·6H2O) was purchased from Alfa Aesar (Johnson Matthey, USA). Disodium hydrogen phosphate (Na2HPO4), sodium dihydrogen phosphate (NaH2PO4), sodium hydroxide (NaOH), polyvinylpyrrolidone, cyclodextrin, sodium dodecylsulfate, sodium carboxymethyl cellulose, tween 80, n-propyl alcohol and iso-propyl alcohol were all of analytical reagent grade and were purchased from Beijing Chemical Factory (Beijing, China). Standard substances of Chlorpromazine, chlorpromazine sulfoxide, 2-chlorophenothiazine and demethylchlorpromazine were purchased from National Institutes for Food and Drug Control (Beijing, China).
2.2. Solutions preparation

Ru(bpy)$_3^{2+}$ solutions were prepared with Ru(bpy)$_3$Cl$_2$·6H$_2$O and secondary distilled water. Phosphate buffer solutions (PBS) were prepared with disodium hydrogen phosphate, sodium dihydrogen phosphate and secondary distilled water. NaOH solution was prepared with NaOH and secondary distilled water. Standard solutions of CPZ, CPZSO, 2-CPTZ and DMCPZ were prepared with their standard substances and secondary distilled water. All solutions used in the experiment must be filtered through a 0.22 μm cellulose acetate membrane.

2.3 Apparatus

CE-ECL was performed on a MPI-B multi-parameter chemiluminescence analysis test system (Xi’an Remex analytical instruments Co., Ltd., Xi’an, China). Cyclic voltammetry and potentiostatic method were carried out in a three electrodes system with a platinum working electrode of 500 μm in diameter, an Ag/AgCl reference electrode of 300 μm in diameter and a platinum wire auxiliary electrode of 1 mm in diameter. Uncoated capillary (25μm x 40 cm, Yongnian Optical Fiber Factory, Hebei, China) was rinsed respectively with 0.1 mol/L NaOH solution for 20 min, secondary distilled water for 10 min and running buffer for 15 min before use.

3. RESULTS AND DISCUSSION

3.1 CEL detection conditions

The optimization of detection conditions is similar to our previous work [17]. Optimized detection parameters are 1.15 V (vs. Ag/AgCl) of detection potential and 45mmol/L of phosphate in buffer solution (pH 6.5) containing 5 mmol/L Ru(bpy)$_3^{2+}$ in CEL detection cell. The optimization process is detailed in Electronic Supplementary Material.

3.2 CE separation conditions

3.2.1 Selection of separation buffer

Compared with CPZ, DMPZ does not have the methyl bonded to amine. The similar structure of CPZ and DMPZ make them difficult to separate by ordinary electrophoresis. The electrophoresis peaks of CPZ and DMPZ overlapped when the separation buffer did not contain additives like many conventional methods [2, 4-6]. Additives in separation buffer have a great influence on the separation of target compounds [1, 3]. In order to obtain good resolution, the effect of polyvinylpyrrolidone, cyclodextrin, sodium dodecylsulfate, Sodium carboxymethyl cellulose, Tween 80, n-propyl alcohol and iso-propyl alcohol on the separation of CPZ and DMPZ were studied by adding them into phosphate buffer as separation buffer, respectively. The results showed that polyvinylpyrrolidone,
cycloexextrin and tween 80 had little effect, sodium dodecylsulfate and sodium carboxymethyl cellulose had obvious influence, and n-propyl alcohol and iso-propyl alcohol had great influence on their separation. The effect of iso-propyl alcohol is particularly prominent, which can distinctly improve the separation of CPZ and DMPZ. The effect of iso-propyl alcohol volume fraction in separation buffer on separation was further studied. The results showed that CPZ and DMPZ could be separated completely when the volume fraction of iso-propyl alcohol in separation buffer was 17.5%. In following experiments, 17.5% iso-propyl alcohol aqueous solution (v/v) containing phosphate was used as the separation buffer.

3.2.2 Injection voltage and time

The sample volume of CE is the electrokinetic injection volume, which is proportional to the injection voltage and injection time. The sample volume directly affects ECL intensity. The injection time was fixed at 10 s, and the effect of inject voltage from 4-20 kV on the ECL intensities of CPZ and DMCPZ was studied in detail. The results show that their ECL intensities increased sharply before 11 kV and tardily later with the increase of the injection voltage and the reproducibility gradually deteriorated after 16 kV. The injection voltage was fixed at 12kV, and the effect of inject time ranging from 2-20 s on the ECL intensities of CPZ and DMCPZ was studied. The results show that the ECL intensities increased sharply for DMCPZ before 6s and for CPZ before 7 s and tardily for both of them later with the increase of the injection time. However, too-long injection time will introduce more analytes into the capillary, which will lead to poor resolution and even overload. Therefore, to compromise between the ECL intensity and the separation effect, 5-8 s and 11-16 kV were chosen as the optimized injection time and injection voltage, respectively. This is somewhat different from the literature work. Most of the injection time in the literature is longer than 8 s, such as 10 s [1-5].

3.2.3 Separation voltage

The influence of the separation voltage on the ECL intensities of CPZ and DMCPZ was systematically investigated over the range of 8–20 kV. For both of them, ECL intensities increased with separation voltage from 8 to 14 kV, and then they slowly decreased from 14 to 20 kV. The baseline noise significant increase when the separation voltage was higher than 14 kV. On the one hand, the Joule heat increased with the increase of separation voltage will make the noise larger. On the other hand, the strong flow of effluent from the capillary may reduce the concentration of Ru(bpy)$_3^{2+}$ on the electrode surface to result in reducing the efficiency of light emitting. In order to obtain high ECL intensity and short migration time, the separation voltage should not exceed 14 kV, which seems to be more than 14 KV in most literatures [2, 4-6].

3.2.4 The pH of separation buffer and concentration of phosphate

The pH of separation buffer affects the electro osmotic flow (EOF) and the extent of ionization
of each analyte in the capillary, which determine the migration time, the resolution and the sensitivity of the analytes. The results indicated that CPZ and DMCPZ could not be separated completely in electropherogram when pH was higher than 6. The effect of concentration of phosphate in separation buffer was also investigated. The results showed that the resolution is not improved significantly when the concentration of phosphate increasing from 5 to 24 mmol/L, and the separation effect becomes worse, the sample migration time gradually prolongs and the baseline is unstable when the concentration of phosphate is higher than 24 mmol/L. This was ascribed to the increased Joule heat caused by the increased ionic strength. In order to obtain ideal separation and short migrating time, smaller buffer pH than 6 and lower phosphate concentration than 24 mmol/L are necessary. The work in the literature seems to have been done more under weak alkaline conditions, such as pH 8 [1, 3-4, 6].

![Electrophoretogram of mixture of the standard CPZ, CPZSO, 2-CPTZ and DMCPZ.](image)

**Figure 2.** Electrophoretogram of mixture of the standard CPZ, CPZSO, 2-CPTZ and DMCPZ.

### 3.2.5 Separation conditions

The overall optimization of separation conditions is especially important when multiple components are simultaneously determined. The experimental results show that the migration times and the peak profiles are the main factors of influencing the separation of components. The migration time mainly depends on the electrophoresis ionic strength and the separation voltage. The electrophoresis ion strength can be appropriately changed by adjusting the concentration of phosphate and pH value of the separation buffer. The component peak profile is related to the sampling volume and the migration time of the component. The sampling volume can be changed by adjusting the injection voltage and injection time. Although the long migration time is beneficial to the separation of components, it is easy to cause the peak broadened and the column effect decreased. Considering these conditions, the electrophoresis separation diagram of a mixed solution comprising CPZ, CPZSO, 2-CPTZ and DMCPZ (see figure 2) was obtained through a large number of comprehensive optimization experiments. The separation conditions were determined: separation buffer of 17.5% iso-propyl...
alcohol aqueous solution (v/v) containing 21mmol/L phosphate (pH 5.3), separation voltage of 13.1 kV, sample injection time of 6 s and sample injection voltage of 11.5 kV. It can be seen that CPZ and DMCPZ can be completely separated under these conditions.

3.3 Method performances

The optimized CE–ECL detection conditions were as follows: detection potential at 1.15 V (vs. Ag/AgCl), 5 mmol/L Ru(bpy)32+ and 45 mmol/L PBS at pH 6.5 in the detection cell, 17.5% iso-propyl alcohol aqueous solution (v/v) containing 21mmol/L phosphate at pH=5.3 as separation buffer, electrokinetic injection 6 s at 11.5 kV, separation voltage at 13.1 kV. Under the optimal conditions, the CE–ECL method was successfully applied for the separation and detection of CPZ and DMCPZ. The performances of analytical method were summarized in Table 1.

Table 1. Regression equation, repeatability and limit of detection.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Regression Equation</th>
<th>Linear Range/(g/L)</th>
<th>RSDI/%</th>
<th>RSDt/%</th>
<th>Limit of Detection /(g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPZ</td>
<td>I = 431.2C+ 185.3</td>
<td>2.6×10⁻⁶~5.8×10⁻³</td>
<td>2.2</td>
<td>1.1</td>
<td>8.1×10⁻⁷</td>
</tr>
<tr>
<td>DMCPZ</td>
<td>I = 196.8C+ 97.4</td>
<td>3.0×10⁻⁵~7.5×10⁻³</td>
<td>2.5</td>
<td>1.0</td>
<td>8.9×10⁻⁶</td>
</tr>
</tbody>
</table>


3.4 Sample analysis

The 25 mg of CPZ was mixed into the food to feed the healthy dogs of 18-20 kg, and collected its urine. After filtration, the urine sample is determined directly by the method described above. The results are shown in Figure 3. The component peaks appeared more in Figure 3 than in Figure 2.

![Figure 3. Electrophoretogram of urine sample original liquid.](image)
This indicates that not only CPZ, CPZSO, 2-CPTZ and DMCPZ, but also other components in urine sample are detected simultaneously.

In order to further determine the peak locations of the targets, the CE-ECL measurement of a mixture solution of containing 1 ml standard solution (5×10^{-6} mg/mL CPZ, 1×10^{-5} mg/mL CPZSO, 5×10^{-6} mg/mL 2-CPTZ and 1×10^{-5} mg/mL DMCPZ) and 5 ml urine sample was performed. The results were shown in figure 4.

Compared with Figure 3, the luminescence intensity of the four peaks in figure 4 is significantly increased, which can accurately determine the peak locations of CPZ, CPZSO, 2-CPTZ and DMCPZ. Miscellaneous peaks in electrophoretogram should be electrochemical luminescence signals of other metabolites.

![Electrophoretogram of urine sample spiked with standards.](image)

**Figure 4.** Electrophoretogram of urine sample spiked with standards.

### 4. CONCLUSION

In this paper, a new analytical method based on CE–ECL for the determination of CPZ, CPZSO, 2-CPTZ and DMCPZ is proposed, especially for the optimization of separation conditions of compounds CPZ and DMCPZ which could not be distinguished by conventional methods without additives. All of the four targets can be separated well within 16 min, with high sensitivity and good reproducibility. In addition, it is an effective analytical method for routine study of antihistamines and their metabolites in urine.

**ACKNOWLEDGEMENTS**

This work was supported by Beijing Natural Science Foundation of China (Grant No.2152013) and State 863 Program of China (2015AA020200).
1. Concentration of Ru(bpy)$_3^{2+}$

Ru(bpy)$_3^{2+}$ was used as the ECL reagent in the system and its concentration had a great influence on the ECL signal. The ECL intensity increased obviously with increasing the concentration of Ru(bpy)$_3^{2+}$ in the range from 1.0 to 11.0 mM due to yield more electrogenerated excited state species [Ru (bpy)$_3^{2+}$]* which can emit rays on falling to the ground state in detection pool. The background signals, however, increased markedly when its concentration exceeded 6 mM. To get a high S/N value, high ECL efficiency, and a moderate reagent consumption, 5 mM Ru(bpy)$_3^{2+}$ was chosen in the experiment. After an operation for 2 h, it was needed to replenish the Ru(bpy)$_3^{2+}$ solution to eliminate the change of concentration of Ru(bpy)$_3^{2+}$ and maintain good reproducibility.

2. Detection potential

The ECL intensity is dependent on the rate of the light-emitting chemical reaction, and this reaction rate relies on the detection potential. Therefore, the detection potential should be investigated to obtain a high sensitivity. As shown in Fig. 1S, the effect of detection potential on ECL intensities of CPZ, CPZSO, 2-CPTZ and DMCPZ was investigated in the range of 0.8–1.3 V. The four analytes had similar behavior of the ECL intensity vs. voltage. Their ECL intensities were very weak when the detection potential was lower than 1.0V, because Ru(bpy)$_3^{2+}$ can not be oxidized at this potential. The ECL intensities firstly increased and then decreased with the detection potential from 1.0 to 1.3 V. The ECL signals reached maximum at 1.15V for CPZ and 2-CPTZ, 1.15-1.20V for CPZSO and DMCPZ, respectively. Hence, the detection potential was set at 1.15 V by comprehensive consideration.

Fig. 1S. Effect of detection potential on ECL intensity
3. pH of buffer in ECL cell

Since Ru(bpy)$_3^{2+}$ ECL reaction with alkylamine depends on the buffer pH value to a great extent, the influence of pH value of the detection buffer on ECL intensity was evaluated by varying the pH values of phosphate buffer solution (PBS) in the range of 4.0–9.0. As shown in Fig. 2S, the ECL intensities increased with pH value from 4.0 to 6.0 and then decreased at higher pH value than 7.0. The reason might be the competition of the reaction of Ru(bpy)$_3^{3+}$ with OH$^-$ ions at higher pH values. The maximum ECL intensities appeared at pH 5.5-6.5 for CPZ and 2-CPTZ, 6.5-7.0 for CPZSO and 6.0-7.0 for DMCPZ. Therefore, PBS at pH 6.5 was used as the detection buffer in our experiment.

![Fig. 2S. Influence of pH of phosphate-buffer in ECL cell on ECL intensity](image)

4. Concentration of PBS in ECL cell

![Fig. 3S. Influence of concentration of phosphate-buffer in ECL cell on ECL intensity](image)
The buffer concentration in the detection cell was also found to affect the ECL intensity. As shown in Fig. 3S, the maximum ECL intensity appeared at 40-45 mmol/L for CPZ and 2-CPTZ, 45-50 mmol/L for CPZSO and 40-50 mmol/L for DMCPZ. Therefore, the buffer concentration of 45 mmol/L was used as the detection buffer in our experiment.

References


© 2019 The Authors. Published by ESG (www.electrochemsci.org). This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution license (http://creativecommons.org/licenses/by/4.0/).