Simultaneous Determination of Promethazine and its Metabolites by Improved Capillary Electrophoresis Coupled with Electrochemiluminescence

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Promethazine sulfoxide (PMZSO), dioxopromethazine (DOPMZ) and demethylpromethazine (DMPMZ) are all the main metabolites of promethazine (PMZ) in vivo. DMPMZ has only one methyl less than PMZ. The structural difference between PMZ and DMPMZ is too small to separate them by conventional capillary electrophoresis (CE). However, the separation efficiency of PMZ and DMPMZ can be improved by adjusting the composition of separation buffer. Combined with electrochemiluminescence (CEL) detection technology, a simultaneous determination method of PMZ, PMZSO, DOPMZ and DMPMZ by improved CE coupled with end-column CEL was established. The parameters about CE separation and CEL detection were investigated in detail. The optimum experimental conditions were detection potential 1.20 V (vs. Ag/AgCl), 40 mmol/L of phosphate buffer solution (pH 6.5) containing 6 mmol/L Ru(bpy)32+ in CEL detection cell, separation buffer of 21.5% iso-propyl alcohol aqueous solution (v/v) containing 20 mmol/L phosphate (pH 5.0), separation voltage of 14 kV, sample injection time of 7 s and sample injection voltage of 12 kV. The limits of detection (3σ) of this method were 7.3×10⁻⁷ mg/mL for PMZ, 8.5×10⁻⁶ mg/mL for PMZSO, 1.5×10⁻⁶ mg/mL for DOPMZ and 4.7×10⁻⁶ mg/mL for DMPMZ. The relative standard deviations were less than 2.8% for ECL intensity and less than 1.1% for migration time. This method was successfully utilized to simultaneously determine PMZ, PMZSO, DOPMZ and DMPMZ in whole urine sample of pet dog.

Keywords: Improved capillary electrophoresis; Electrochemiluminescence; Promethazine; Metabolite; Urine sample
1. INTRODUCTION

Promethazine (PMZ) is widely used as an antihistamine to alleviate allergic symptoms and enhance the analgesic, anesthetic and sedative effects of other drugs [1]. In vivo, PMZ can be biotransformed into a variety of metabolites which most belong to the phenothiazine category, such as promethazine sulfoxide (PMZSO), demethyl promethazine (DMPMZ), dioxopromethazine (DOPMZ) and so on. Simultaneous determination of PMZ and its metabolites in urine is extremely important to realize the economic effect and pharmacokinetics of PMZ.

Numerous methods have been employed to analyze PMZ and its metabolites, such as spectrophotometry [2, 3], electrophoresis [4], chromatography [5–7], electrochemical methods [8-11] and fluorescence [12].

For the past few decades, Tris (2,2’-bipyridyl) ruthenium (II) (Ru(bpy)₃²⁺)-based electrochemiluminescence (ECL) has attracted wide attention owing to its inherent high sensitivity, selectivity and stability. Capillary electrophoresis (CE) is a promising high-performance biochemical and medical analysis method with the advantages of strong separation ability, short analysis time and less sample consumption. CE separation with end-column ECL detection (CE-ECL) have been widely studied and used to analyze various drugs [13–29], antibiotics [30–32], enzymes [33], alkaloids [34–37], amines [38–42], hormones [43] and pesticide residues [44,45] in different foods, pharmaceuticals, animals and plants. Continuous determinations of four or more components are rarely reported.

PMZ and DMPMZ are very similar in structure, which is difficult to distinguish in our previous work [29]. In this paper, PMZ, PMZSO, DOPMZ and DMPMZ with similar structure (see Fig. 1) were separated and detected simultaneously in the whole urine by comprehensive optimization of CE-ECL conditions. The results show that the present method is sensitive and reliable for the simultaneous determination of PMZ and its metabolites for the study of pharmacokinetics, pharmacodynamics and safety evaluation, rational dose design and potential mechanism.

![Figure 1. Structure of promethazine, promethazine sulfoxide, dioxopromethazine and demethyl promethazine.](image-url)

2. EXPERIMENTAL

2.1. Materials and Reagents

Tris (2,2’-bipyridyl) ruthenium(II) dichloride hexahydrate (Ru(bpy)₃Cl₂·6H₂O) was purchased
from Alfa Aesar (Johnson Matthey, USA). Disodium hydrogen phosphate (Na$_2$HPO$_4$), sodium dihydrogen phosphate (NaH$_2$PO$_4$), sodium hydroxide (NaOH), polyvinylpyrrolidone, cyclodextrin, sodium dodecysulfate, 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 promethazine, promethazine sulfoxide, demethyl promethazine, dioxopromethazine 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 promethazine, promethazine sulfoxide, dioxopromethazine, demethyl promethazine 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 filtration 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 performed in a three electrodes system consisting of a working electrode with a diameter of 500 μm platinum, a reference electrode with a diameter of 300 μm Ag/AgCl and an auxiliary electrode with a diameter of 1 mm platinum wire. Uncoated capillary (25 μm 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. Optimization of detection conditions

3.1.1 Concentration of Ru(bpy)$_3^{2+}$

As an ECL reagent, Ru(bpy)$_3^{2+}$ is oxidized to Ru(bpy)$_3^{3+}$ on the working electrode, and then reacts with reductive coexistences (such as amines) to produce the excited state product [Ru(bpy)$_3^{2+}$]$^*$ which emits photons when it returns to the ground state. It can be seen that the initial concentration of Ru(bpy)$_3^{2+}$ has a great influence on the ECL intensity. The ECL intensity increased obviously with increasing the concentration of Ru(bpy)$_3^{2+}$ in the range from 1.0 to 11.0 mmol/L 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 mmol/L. To get a high S/N value and high ECL efficiency, 6 mmol/L 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.

![Figure 2. Effect of detection potential on ECL intensity.](image)

3.1.2 Detection potential

The ECL intensity is dependent on the rate of chemiluminescence (CL) reaction, and the formation rate of reactant of CL reaction relies on the detection potential in this system. Therefore, the detection potential should be investigated to obtain a high sensitivity. As shown in Fig. 2, the effect of detection potential on ECL intensities of PMZ, PMZSO, DOPMZ and DMPMZ was studied 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 1.0 V, because Ru(bpy)$_3^{2+}$ could 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 1.15 V for PMZ, 1.15–1.20 V for DOPMZ and 1.2 V for PMZSO and DMPMZ, respectively. The ECL signals are almost the same at 1.15 V and 1.20 V for PMZ. Therefore, 1.20 V was selected as the detection potential by comprehensive consideration.

3.1.3 pH of buffer in ECL cell

Since Ru(bpy)$_3^{2+}$ ECL reaction with alkylamine is greatly affected by pH value, 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. 3, 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. As shown in
Fig. 3, the maximum ECL intensities appeared at pH 6.0-6.5 for PMZ and DOPMZ, 6.5-7.0 for PMZSO and 5.5-7.0 for DMPMZ. Therefore, PBS at pH 6.5 was used as the detection buffer in our experiment. The pH values of PBS in the literature [13-18] are mostly 6.0-7.0, which is consistent with our results.

Figure 3. Influence of pH of phosphate-buffer in ECL cell on ECL intensity.

3.1.4 Concentration of PBS in ECL cell

The buffer concentration in the detection cell was also found to affect the ECL intensity. As shown in Fig. 4, the maximum ECL intensity appeared at 40-45 mmol/L for PMZ and DOPMZ, 35-40 mmol/L for PMZSO and 35-50 mmol/L for DMPMZ. Therefore, 40 mmol/L PBS was used as the detection buffer in our experiment.

Figure 4. Influence of concentration of phosphate-buffer in ECL cell on ECL intensity.
3.2. Optimization of separation parameters

3.2.1 Selection of separation buffer

The difference between PMZ and DMPMZ is only that the nitrogen atom at the end is bonded to two methyl groups or one methyl group. The structure difference between PMZ and DMPMZ is too small to be separated by general electrophoresis. When the separation buffer did not contain additives like many conventional methods [13, 16-18], the electrophoretic peaks of PMZ and DMPMZ overlap and cannot be separated. In order to obtain good separation, the effect of polyvinylpyrrolidone, cyclodextrin, sodium dodecylsulfate, sodium carboxymethyl cellulose, tween 80, n-propyl alcohol and iso-propyl alcohol on the separation of PMZ and DMPMZ were studied by adding them into the separation buffer, respectively. The results showed that polyvinylpyrrolidone, cyclodextrin and tween 80 had no obvious influence, sodium dodecylsulfate and sodium carboxymethyl cellulose had obvious influence, and n-propyl alcohol and iso-propyl alcohol had great influence on the separation effect. Iso-propyl alcohol is especially effective in improving the separation of PMZ and DMPMZ. The influence of iso-propyl alcohol volume fraction in separation buffer on separation was studied in detail. The results showed that PMZ and DMPMZ could be separated completely when the volume fraction of iso-propyl alcohol in separation buffer was 21.5%. In following experiments, 21.5% isopropanol aqueous solution (v/v) containing phosphate was used as the separation buffer.

3.2.2 Injection voltage and time

Capillary electric injection volume is proportional to the injection voltage and injection time, and sample injection volume directly influence ECL intensity. The effects of injection voltage from 4-20 kV on the ECL intensities of PMZ, PMZSO, DOPMZ and DMPMZ were studied in detail by fixing the injection time at 10 s. The results show that the ECL intensities of the four molecules increase sharply before 11 kV with the increase of injection voltage, then slowly, and the reproducibility deteriorate gradually after 16 kV. The effects of inject time from 2-20 s on the ECL intensities of PMZ, PMZSO, DOPMZ and DMPMZ were studied by fixing the injection voltage at 12 kV. The results show that the ECL intensities increase sharply for PMZSO and DMPMZ before 5 s, and for DOPMZ and PMZ before 6 s with the increase of the injection time, and then slowly for all of them.

However, too long injection time can lead to lower resolution, because more analytes are introduced into capillaries, and even overload occurs. Therefore, 6-10 s and 11-16 kV are selected as injection time and injection voltage respectively in order to take into account the ECL intensity and separation effect.

3.2.3 Separation voltage

The influence of the separation voltage on the ECL intensities of PMZ, PMZSO, DOPMZ and DMPMZ was studied on the range of 8–20 kV. For all of them, ECL intensities increased with separation voltage from 8 to 15 kV, and then they slowly decreased from 15 to 20 kV. The baseline
noise significant increase when the separation voltage was higher than 15 kV. On the one hand, the Joule heat increased with the increase of separation voltage which will make the noise enlarge. 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. To obtain a high ECL intensity and short migration time, the separation voltage should not exceed 15 kV, which seems to be more than 15 kV in most literatures [14, 16-18].

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 determines the migration time, the resolution and the sensitivity of the analytes. The results indicated that when pH was higher than 6, PMZ, PMZSO, DOPMZ and DMPMZ could not be separated completely in electropherogram. The effect of concentration of phosphate in separation buffer was also investigated. The results showed that the resolution was not improved with the increasing of concentration of phosphate from 5–24 mmol/L, and the separation was worse, the migration time of analytes prolonged gradually and the baseline became unstable when the concentration of phosphate was higher than 24 mmol/L. This was ascribed to the increased Joule heating caused by the increased ionic strength. In order to obtain ideal separation and shorter migrating time, smaller buffer pH than 6 and lower buffer concentration than 24 mmol/L are necessary. The studies in the literature are sometimes carried out under weak alkaline conditions, such as pH 8 [13, 15, 18].

3.2.5 Separation conditions

When multi-components are simultaneously determined, the overall optimization of separation conditions is particularly important.

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 and pH value of the buffer solution. 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 PMZ, PMZSO, DOPMZ and DMPMZ (see figure 5) was obtained through a large number of comprehensive optimization experiments. The separation conditions were determined: separation buffer of 21.5% iso-propyl alcohol aqueous solution (v/v) containing 20 mmol/L phosphate (pH 5.0), separation voltage of 14 kV, sample injection time of 7 s and sample injection voltage of 12 kV. It can be seen that PMZ and DMPMZ can be completely separated under these conditions. These separation conditions are quite different from those reported in
Figure 5. Electrophoretogram of mixture of the standard PMZ、PMZSO、DOPMZ and DMPMZ.

3.3 Method performances

The optimized CE–ECL detection conditions were as follows: detection potential at 1.2 V (vs. Ag/AgCl), 6 mmol/L Ru(bpy)$_3^{2+}$ and 40 mmol/L PBS at pH 6.5 in the detection cell, 21.5% iso-propyl alcohol aqueous solution (v/v) containing 20 mmol/L PBS at pH=5.0 as separation buffer, electrokinetic injection 7 s at 12 kV, separation voltage at 14 kV. Under the optimal conditions, the CE–ECL method was successfully applied for the separation and detection of PMZ、PMZSO、DOPMZ and DMPMZ. The analytical results were summarized in Table 1.

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

<table>
<thead>
<tr>
<th>Drug</th>
<th>Regression Equation</th>
<th>Linear Range/(g/L)</th>
<th>RSD$_I$/%</th>
<th>RSD$_t$/%</th>
<th>Limit of Detection/(g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMZ</td>
<td>$I = 413.7C + 157.5$</td>
<td>$3.5 \times 10^{-6} \sim 6.6 \times 10^{-3}$</td>
<td>2.8</td>
<td>1.05</td>
<td>$7.3 \times 10^{-7}$</td>
</tr>
<tr>
<td>PMZSO</td>
<td>$I = 224.8C + 107.7$</td>
<td>$9.1 \times 10^{-5} \sim 7.6 \times 10^{-3}$</td>
<td>2.0</td>
<td>1.1</td>
<td>$8.5 \times 10^{-6}$</td>
</tr>
<tr>
<td>DOPMZ</td>
<td>$I = 705.1C - 65.3$</td>
<td>$9.3 \times 10^{-6} \sim 5.7 \times 10^{-3}$</td>
<td>1.3</td>
<td>0.95</td>
<td>$1.5 \times 10^{-6}$</td>
</tr>
<tr>
<td>DMPMZ</td>
<td>$I = 194.7C + 477.5$</td>
<td>$3.1 \times 10^{-5} \sim 8.6 \times 10^{-3}$</td>
<td>2.4</td>
<td>1.0</td>
<td>$4.7 \times 10^{-6}$</td>
</tr>
</tbody>
</table>

3.4 Sample analysis

The 25 mg of PMZ 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 6. The component peaks appeared more in figure 6 than in figure 5. This indicates that not only PMZ, PMZSO, DOPMZ and DMPMZ, but also other components are detected simultaneously.

![Electrophoretogram of urine sample original liquid.](image1)

**Figure 6.** Electrophoretogram of urine sample original liquid.

![Electrophoretogram of urine sample spiked with standards.](image2)

**Figure 7.** Electrophoretogram of urine sample spiked with standards.
In order to further determine the peak location of the target, the CE-ECL measurement of a mixture solution of containing 1 ml standard solution (5×10^(-6) mg/mL PMZ, 5×10^(-5) mg/mL PMZSO, 1×10^(-4) mg/mL DOPMZ and 1×10^(-5) mg/mL DMPMZ) and 5 ml urine sample was performed. The results were shown in figure 7.

Compared with figure 6, the luminescence intensity of the four peaks in figure 7 is significantly increased, which can accurately determine the location of PMZ, PMZSO, DOPMZ and DMPMZ. Miscellaneous peaks should be electrochemical luminescence signals for other metabolites.

4. CONCLUSION

This work demonstrated a new analytical procedure based on CE–ECL for simultaneous determination of PMZ, PMZSO, DOPMZ and DMPMZ by improved CE coupled with end-column CEL. The four analytes could be well separated within 14 min with high sensitivity, wide linear range, and good reproducibility by comprehensive optimization of CE-ECL conditions. It was firstly utilized to directly simultaneously detect PMZ, PMZSO, DOPMZ and DMPMZ in urine sample of pet dog. In addition, it is an efficient approach for the routine study of antihistamine drug and its metabolites in urine.

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References

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