Optimization of Capillary Electrophoresis Separation Conditions of Chlorpromazine, Promethazine and Their Main Metabolites by RSM

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It is difficult to separate multiple phenothiazines with similar structures by capillary electrophoresis. However, under the experimental conditions optimized by response surface methodology (RSM), ten similar molecules of chlorpromazine, promethazine and their metabolites can be well separated in our experiment. Among them, the resolution of two difficult-to-separate molecules (chlorpromazine sulfoxide and hydroxychlorpromazine) is 1.39 (incomplete separation) under single factor experimental conditions and 1.51 (complete separation) under optimized experimental conditions. The optimum values of pH of phosphate buffer solution (PBS), concentration of NaCl in PBS, concentration of tween 40 in PBS and separation voltage were 6.75, 49.06 mmol/L, 26.58 mmol/L and 16.86 kV, respectively. All of the ten phenothiazines have good linear relationship. The recoveries of standard addition for dog urine samples without pretreatment were 87.3%-112.6%.

Keywords: Capillary electrophoresis, Chlorpromazine, Promethazine, Metabolite, Response surface methodology

1. INTRODUCTION

Both chlorpromazine (CPZ) and promethazine (PMZ) are important phenothiazines clinical drugs. CPZ with special central nervous system inhibition is mainly used in the treatment of schizophrenia, mania and so on [1, 2]. PMZ, which not only has strong anti allergic and central nervous system stabilization [3, 4], but also can improve the analgesic, anesthetic and sedative effects [5], is mainly used in the treatment of allergic diseases. Mixing the two drugs can reduce the activity of dopamine in central nervous system. Clinical studies have also shown that the mixed use of chlorpromazine and promethazine has neuroprotective effects on patients with intracerebral hemorrhage and subarachnoid hemorrhage [6, 7]. The main metabolites of CPZ are demethylchlorpromazine (DMCPZ), chlorpromazine sulfoxide (CPZSO), demethylchlorpromazine sulfoxide
(DMCPZSO) and hydroxychlorpromazine (HCPZ). The main metabolites of PMZ are promethazine sulfoxide (PMZSO), demethylpromethazine (DMPMZ), dihydroxydemethyl promethazine (DHDMPMZ) and dihydroxypromethazine (DHPMZ). Therefore, the establishment of rapid determination methods for CPZ, PMZ and their main metabolites is of great significance for the study of the dose, utilization rate and metabolic pathway of these drugs.

Capillary electrophoresis (CE) is a rapid, economical and efficient separation method. Electrochemiluminescence (ECL) based on tris (2,2'-bipyridyl) ruthenium (II) (Ru(bpy)_3^{2+}) is an attractive analytical method with high sensitivity, selectivity and repeatability for organic amine. CE separation couple with ECL analysis has been widely used to analyze various drugs [8-21], enzymes [22, 23], alkaloids [24-28], antibiotics [29-33] and pesticide residues [34-36]. Most of phenothiazines and their main metabolites contain secondary amine group or tertiary amino group. They can obviously enhance the ECL signal of Ru(bpy)_3^{2+}. It is a good attempt to separate and analyze them with CE-ECL.

The molecular structures of CPZ, PMZ and their metabolites are very similar, and it is difficult to completely separate them under conventional CE conditions. In our previous work, we used CE-ECL method to analyze two or four phenothiazines [9, 37]. This paper attempts to optimize the separation conditions by response surface methodology (RSM), a widely used multi parameter statistical optimization method [38-41], in order to separate and analyze 10 phenothiazine compounds such as CPZ, PMZ and their metabolites.

2. EXPERIMENTAL

2.1. Materials and drugs

Tris (2, 2’-bipyridyl) ruthenium (II) dichloride hexahydrate (Ru(bpy)_3Cl_2·6H_2O) was purchased from Alfa Aesar (Johnson Matthey, USA). Standard substances of chlorpromazine (CPZ), promethazine (PMZ), demethylchlorpromazine (DMCPZ), chlorpromazine sulfoxide (CPZSO), demethylchlorpromazine sulfoxide (DMCPZSO), hydroxychlorpromazine (HCPZ), promethazine sulfoxide (PMZSO), demethylpromethazine (DMPMZ), dihydroxydemethyl promethazine (DHDMPMZ) and dihydroxypromethazine (DHPMZ) were purchased from National Institutes for Food and Drug Control (Beijing, China). Disodium hydrogen phosphate (Na_2HPO_4), sodium dihydrogen phosphate (NaH_2PO_4), sodium hydroxide (NaOH), hydrochloric acid (HCl), sodium chloride (NaCl) and tween 40 were all of analytical reagent grade and were purchased from Beijing Chemical Reagent Company (Beijing, China).

2.2. Apparatus and conditions

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. Capillary (25 μm x 40 cm) 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. See our previous work [9] for details.

ECL conditions: Detection potential is 1.15 V (vs. Ag/AgCl). Concentration of Ru(bpy)$_3^{2+}$ is 6 mmol/L. Concentration of PBS (pH 6.8) in test cell is 40 mmol/L.

3. RESULTS AND DISCUSSION

3.1. Selection of capillary electrophoresis parameters

CE separation conditions, such as pH of PBS, ionic strength of PBS, additives in PBS, and separation voltage, have a great impact on the separation of the analytes. In this part, taking the resolution of adjacent analytes flowing out of capillary as the test index, the effects of different capillary electrophoresis parameters on their separation were studied. The resolution (R) is the ratio of the difference of retention time ($t$) between two adjacent components and the average width of peak base ($Y$) of the two components. Its mathematical expression is as follows:

$$R = \frac{t_2 - t_1}{(Y_1 + Y_2) / 2}$$

It is generally believed that the two components can be completely separated from the shape of the peaks when their resolution is not less than 1.5. See our previous work for detailed calculation [42].

![Figure 1. Effects of pH of phosphate buffer solution on the resolutions of ten phenothiazines at separation voltage 18 kV.](image)
3.1.1 The pH of PBS

The pH of PBS is an important factor affecting the separation effect. In the literature work of CE with PBS medium, the application range of pH value is mostly between 5-9 [8-15, 21-25, 31-33]. Therefore, we mainly study the influence of pH in this range on the resolution. When the pH of the PBS changes from 5.0 to 9.0, the resolutions of 9 pairs of analytes are shown in Figure 1. With the increase of the pH of PBS, the resolutions of 9 pairs of analytes shows a trend of "first increase and then decrease", and reaches the maximum at pH = 7.0-8.0. At pH 7.0, the resolutions of 7 pairs of analytes reached the maximum value, so we chose 7.0 as the pH value of separation buffer solution.

3.1.2 Ionic strength of PBS

The ionic strength of PBS is another important factor affecting the separation efficiency. In general, the ionic strength of PBS can be adjusted by adding a strong electrolyte. In this part, we change the ionic strength of PBS by adding different concentrations of sodium chloride (NaCl), and investigate the effect of ionic strength on the separation. The results are shown in Figure 2. With the increase concentration of NaCl, the ionic strength of PBS increased. When the concentration of NaCl is 45 mmol/L, the resolutions of 9 pairs of analytes are relatively large. This conclusion is supported by many literatures [19-23, 25-32].

Figure 2. Effects of ionic strength of PBS (pH 7.0) on the resolutions of ten phenothiazines at separation voltage 18 kV.
3.1.3 Additive in PBS

Pyrrolidone, tween 40, Cyclodextrin, isopropanol and so on are commonly used to change the solution environment of PBS to improve the effect of fine separation of analytes. Our study found that tween 40 has a great influence on the separation of ten phenothiazines. Figure 3 shows the effect of different concentrations of tween 40 on the resolutions of 9 pairs of analytes. As you can see, when the concentration of tween 40 is 25 mmol/L, all of the resolutions of 9 pairs of analytes are relatively large. The use of additives to improve the separation effect has been confirmed by many experiments [16, 20, 26, 31, 33].

![Figure 3](image_url)

**Figure 3.** Effects of tween 40 in PBS (pH 7.0) containing 45 mmol/L NaCl on the resolutions of ten phenothiazines at separation voltage 18 kV.

3.1.4 Separation voltage

The separation voltage directly affects the migration rate of components, and then changes the separation effect of different components. In this experiment, the separation voltages from 15 kV to 19 kV are investigated. The results are shown in Figure 4. Obviously, 17 kV is the best separation voltage. In the literature work, the separation voltage of most experiments is below 20 kV [16-29], which is consistent with our conclusion.

Unfortunately, there are still four pairs of analytes whose resolution does not reach 1.5, indicating that they could not be completely separated under current experimental conditions.
Figure 4. Effects of separation voltage on the resolutions of ten phenothiazines in PBS (pH 7.0) containing 45 mmol/L NaCl and 25 mmol/L tween 40.

3.2 Optimization of capillary electrophoresis parameters

RSM is a statistical optimization method to solve multivariable problems. It uses the reasonable experimental design method to get some data, then uses the multivariate quadratic regression equation to fit the functional relationship between the factors and the response value, finally through the regression equation analysis to seek the optimal process parameters. In this part, on the basis of single factor experiments, RSM is used to investigate the interactions of different factors. It is expected to improve the resolutions of ten phenothiazines by optimizing the experimental conditions.

3.2.1 Box-Behnken test and experimental results

CPZSO and HCPZ have the same molecular weight and similar molecular structure. From the previous experimental results, we can see that under the single-factor experimental conditions, their resolution is only 1.39, indicating that they could not be completely separated.

Therefore, in this part, we take the resolution of CPZSO and HCPZ as the response value. The pH of PBS, concentration of NaCl in PBS, concentration of tween 40 in PBS and separation voltage were used as research factors. According to the results of single factor experiments, four factors and three levels (see table 1) were used to carry out the Box-Behnken test design.
Table 1. Factors and levels of the Box-Behnken test design.

<table>
<thead>
<tr>
<th>Levels</th>
<th>Factors</th>
<th>pH</th>
<th>C_{NaCl} (mmol/L)</th>
<th>C_{Tween 40} (mmol/L)</th>
<th>Separation voltage (kV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1</td>
<td></td>
<td>6.0</td>
<td>40</td>
<td>20</td>
<td>16</td>
</tr>
<tr>
<td>0</td>
<td></td>
<td>7.0</td>
<td>45</td>
<td>25</td>
<td>17</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>8.0</td>
<td>50</td>
<td>30</td>
<td>18</td>
</tr>
</tbody>
</table>

The results of 29 response surface design trials are shown in Table 2. Multifactor regression fitting analysis was carried out with Design Expert software. The regression equation of the effects of pH value (A), NaCl concentration (B), additive concentration (C) and separation voltage (D) on the resolution (Y) is as follows: \[ Y = 1.39 - 0.058A + 0.27B - 0.0042C + 0.0025D - 0.10AB + 0.092AC + 0.14AD - 0.13BC - 0.030BD - 0.23CD - 0.26A^2 - 0.21B^2 - 0.17C^2 - 0.28D^2. \] The model F-value of 3.56 implies the
model is significant. Within the range of levels of the selected factors, the order of effect on the results is: NaCl concentration (B) > pH value (A) > additive concentration (C) > separation voltage (D).

3.2.2 Interaction of different factors on resolution

The 3D surfaces and contours are plotted by Design Expert software, as shown in figure 5 to figure 10. Each figure represents the influence of the interaction of two independent variables on resolution. The 3D surface is steep and the central area of the contour map presents a nearly complete ellipse, as shown in figures 6, 7 and 10, indicating that the interaction between the two factors is obvious. In addition, the six 3D surfaces are convex and open downward, indicating that there is a maximum in the continuous area of the test point.

**Figure 5.** Effect of interaction of pH and ionic strength of PBS on resolutions.

**Figure 6.** Effect of interaction of pH of PBS and concentration of additive on resolutions.
Figure 5 is 3D surface (a) and contour (b) showing the effect of the interaction between pH of PBS and concentration of NaCl in PBS on resolution. With the increase of pH of PBS and concentration of NaCl, the resolution also changed. When the pH of PBS reaches 6.75 and the concentration of NaCl reaches 49.06 mmol/L, the resolution reaches its maximum.

Figure 6 is 3D surface (a) and contour (b) showing the effect of the interaction between pH of PBS and concentration of tween 40 in PBS on resolution. When the pH of PBS reaches 6.75 and the concentration of tween 40 reaches 26.58 mmol/L, the resolution will reach its maximum.

Figure 7 is 3D surface (a) and contour (b) showing the effect of the interaction between pH of PBS and separation voltage on resolutions. As you can see, when the pH of PBS reaches 6.75 and the separation voltage reaches 16.86 kV, the resolution reaches its maximum.

Figure 8. Effect of interaction of ionic strength of PBS and concentration of additive on resolutions.
Figure 8 is 3D surface (a) and contour (b) showing the effect of the interaction between concentration of NaCl and concentration of additive on resolutions. When the concentration of NaCl reaches 49.06 mmol/L and the concentration of additive reaches 26.58 mmol/L, the resolution reaches its maximum.

Figure 9 is 3D surface (a) and contour (b) showing the effect of the interaction between concentration of NaCl and separation voltage on resolutions. When the concentration of NaCl reaches 49.06 mmol/L and the separation voltage reaches 16.86 kV, the resolution reaches its maximum.

Figure 10 is 3D surface (a) and contour (b) showing the effect of the interaction between concentration of additive and separation voltage on resolutions.
concentration of tween 40 and separation voltage on resolutions. When the concentration of tween 40 reaches 26.58 mmol/L and the separation voltage reaches 16.86 kV, the resolution reaches its maximum.

Based on the above analysis, it can be found that the optimum values of pH of PBS, concentration of NaCl in PBS, concentration of tween 40 in PBS and separation voltage were 6.75, 49.06 mmol/L, 26.58 mmol/L and 16.86 kV, respectively, when the maximum resolution of CPZSO and HCPZ is obtained. As can be seen, it is almost impossible to obtain such optimal experimental conditions through a limited number of single-factor experiments. According to the model, the maximum value of resolution was 1.51. This is 8.7% higher than that selected from single factor experiments. The most important thing is that the resolution of the two most difficult-to-separate molecules is more than 1.5, indicating that all components are well separated.

3.3 Methodology

A series of phenothiazines standard solutions were prepared and used as sample solutions for CE separation and ECL determination. The linear relationship, linear range and detection limit of ten phenothiazines were investigated. The results were summarized in Table 1.

Table 1. Regression equation, linear range and detection limit of ten phenothiazines under the optimized CE conditions.

<table>
<thead>
<tr>
<th>Number</th>
<th>Drug</th>
<th>Regression equation*</th>
<th>Linear range/(μg/L)</th>
<th>Detection limit/(μg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CPZ</td>
<td>( I = 131.7C + 22.5 )</td>
<td>1.0-1200</td>
<td>0.5</td>
</tr>
<tr>
<td>2</td>
<td>DMCPZ</td>
<td>( I = 128.2C + 43.1 )</td>
<td>1.0-1200</td>
<td>0.5</td>
</tr>
<tr>
<td>3</td>
<td>CPZSO</td>
<td>( I = 101.7C + 31.3 )</td>
<td>1.0-1200</td>
<td>0.5</td>
</tr>
<tr>
<td>4</td>
<td>DMCPCZSO</td>
<td>( I = 85.8C + 14.8 )</td>
<td>1.0-1200</td>
<td>0.5</td>
</tr>
<tr>
<td>5</td>
<td>HCPZ</td>
<td>( I = 224.5C + 29.6 )</td>
<td>0.5-1000</td>
<td>0.2</td>
</tr>
<tr>
<td>6</td>
<td>PMZ</td>
<td>( I = 114.7C + 38.4 )</td>
<td>0.5-800</td>
<td>0.2</td>
</tr>
<tr>
<td>7</td>
<td>PMZSO</td>
<td>( I = 91.9C + 19.6 )</td>
<td>1.0-1200</td>
<td>0.5</td>
</tr>
<tr>
<td>8</td>
<td>DMPMZ</td>
<td>( I = 110.3C + 21.2 )</td>
<td>1.0-900</td>
<td>0.5</td>
</tr>
<tr>
<td>9</td>
<td>DHDPMZ</td>
<td>( I = 317.5C + 45.0 )</td>
<td>0.3-600</td>
<td>0.1</td>
</tr>
<tr>
<td>10</td>
<td>DHPMZ</td>
<td>( I = 334.8C + 32.9 )</td>
<td>1.0-1200</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* I: ECL intensity (a.u.); C: mass concentration, μg/L.

3.4 Determination of dog urine samples

15 ~ 20 kg healthy dogs were selected, chlorpromazine and promethazine 20 mg each were ground up and mixed in food to feed the dogs. Dog urine was collected after 2 hours, filtered and determined by direct and standard addition.

The residue and recovery of ten phenothiazines in dog urine were studied. The recoveries of
ten phenothiazines are 87.3% - 112.6%, which shows that this method is reliable. The results are shown in Table 2.

Table 2. Analysis results of actual food samples under the optimized CE conditions.

<table>
<thead>
<tr>
<th>Number</th>
<th>Drug</th>
<th>Measured value (μg/L)</th>
<th>Added value (μg/L)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CPZ</td>
<td>176.5</td>
<td>100</td>
<td>89.8</td>
</tr>
<tr>
<td>2</td>
<td>DMCPZ</td>
<td>11.3</td>
<td>100</td>
<td>90.3</td>
</tr>
<tr>
<td>3</td>
<td>CPZSO</td>
<td>7.1</td>
<td>100</td>
<td>88.5</td>
</tr>
<tr>
<td>4</td>
<td>DMCPZSO</td>
<td>4.4</td>
<td>100</td>
<td>103.2</td>
</tr>
<tr>
<td>5</td>
<td>HCPZ</td>
<td>16.6</td>
<td>100</td>
<td>105.7</td>
</tr>
<tr>
<td>6</td>
<td>PMZ</td>
<td>198.0</td>
<td>100</td>
<td>112.6</td>
</tr>
<tr>
<td>7</td>
<td>PMZSO</td>
<td>35.5</td>
<td>100</td>
<td>87.3</td>
</tr>
<tr>
<td>8</td>
<td>DMPMZ</td>
<td>22.8</td>
<td>100</td>
<td>90.5</td>
</tr>
<tr>
<td>9</td>
<td>DHDMPMZ</td>
<td>3.1</td>
<td>100</td>
<td>97.1</td>
</tr>
<tr>
<td>10</td>
<td>DHPMZ</td>
<td>15.4</td>
<td>100</td>
<td>105.3</td>
</tr>
</tbody>
</table>

4. CONCLUSION

This work demonstrated a new analytical procedure for simultaneous determination of CPZ, PMZ, DMCPZ, CPZSO, DMCPZSO, HCPZ, PMZSO, DMPMZ, DHDMPMZ and DHPMZ in dog urine by CE-ECL. The ten phenothiazines could be well separated and detected with high sensitivity, wide linear range, and good reproducibility under optimized experimental conditions. This method can be used to determine phenothiazines in complex solution directly and rapidly.

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References
