Determination of Chlorpheniramine and Its Binding with Human Serum Albumin by Capillary Electrophoresis with Tris(2,2'-bipyridyl)ruthenium(II) Electrochemiluminescence Detection

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Tris(2,2'-bipyridyl)ruthenium(II) electrochemiluminescence (ECL) detection in a capillary electrophoresis separation system was used for the determination of chlorpheniramine (CPM). The experimental conditions, such as the applied potential, separation voltage, injection voltage, injection time and the pH of the separation buffer were considered in detail. The ECL intensity showed two linear responses to CPM, *i.e.*, from 15 μM to 1 mM and from 0.8 μM to 15 μM with a detection limit of 0.5 μM . The binding of CPM with human serum albumin was also monitored using this method and the binding constant was estimated to be $4.1 \times 10^3 \text{ M}^{-1}$.

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Introduction

Chlorpheniramine (CPM), 3-(4-chlorophenyl)-3-(2-pyridyl)-propyl-*N,N*-dimethyl ammonium hydrogen, is one of the most potent antihistaminic agents in the treatment of allergic and vasomotor rhinitis, allergic conjunctivitis, mild urticaria, angioedema and as adjunct therapy in anaphylactic shock.¹ Various methods are available for the determination of CPM, such as HPLC and LC,² ion-selective electrode (ISE),³ and spectrophotometry.⁴ However, in HPLC and LC analysis, tedious sample clean-up procedures, such as extraction and purification, are always required; a drawback of the ISE method is that its response behavior is affected by the high background conductivity of the solution, the electrical properties of the film, and the electrical double-layer capacitance; some reactions are needed for spectrophotometry because of its significant overlapping in the spectra. Therefore, it is necessary to develop a more sensitive and effective method for CPM detection.

In the past two decades, capillary electrophoresis (CE) has become one of the most powerful and simple separation techniques for the analysis of complex mixtures because of its high resolution, short analysis time, and low operational cost. It has been widely used in the analysis of inorganic ions, organic substances, amino acids, DNA, protein, and so on. Various detection methods for CE are available, including UV-visible absorbance,⁵ fluorescence,⁶ mass spectrometry,⁷ electrochemistry⁸ and electrochemiluminescence (ECL).⁹ Among them, ECL, as a special type of chemiluminescence (CL) in that the luminescence reagent is initiated by electrochemical reactions, has become an important and valuable detection technique in analytical chemistry in recent years.^{10,11} The major advantages of ECL are a reduction of interferences from solution impurities and elimination of the need for complicated construction of

postcolumn reactors.¹² Among all of the ECL reagents, $\text{Ru}(\text{bpy})_3^{2+}$ -ECL, as a simple and sensitive detection technique, has been applied to a wide variety of compounds and ions for alkylamines,¹³ amino acids,¹⁴ peptides,¹⁵ DNA,¹⁶ due to its stability in aqueous media and high luminescence efficiency. Considering the tertiary amine structure of CPM, the short analysis time of CE, and the high luminescence efficiency of $\text{Ru}(\text{bpy})_3^{2+}$ -ECL, the detection of CPM by capillary electrophoresis with $\text{Ru}(\text{bpy})_3^{2+}$ -ECL is promising. As far as we know, fewer relative reports can be found.

Therefore, in this work, CE separation with end-column $\text{Ru}(\text{bpy})_3^{2+}$ ECL detection for the quantitative determination of CPM was performed. The experimental conditions, such as the injection voltage, separation voltage, injection time and the pH of the separation buffer were considered in detail. The association of human serum albumin (HSA) with CPM was also realized by combining equilibrium dialysis and CE- $\text{Ru}(\text{bpy})_3^{2+}$ -ECL.

Experimental

Reagents and solutions

All of the reagents used were of analytical grade unless stated otherwise. Tris(2,2'-bipyridyl)ruthenium(II) chloride hexahydrate was obtained from J&K Chemical Company, while HSA and the dialysis bag (14000 Da) were purchased from Sigma, and CPM from Xi'an Pharmaceutical Factory. A stock solution of 0.1 M CPM was prepared in a phosphate buffer solution (PBS) (pH 8.0), and stored at 4°C. A series of standard solutions for the calibration curve and reproducibility were prepared by diluting the solution with PBS just before use. Double-distilled water was used throughout.

Apparatus

CE-ECL was from Xi'an Remax Tech. Ltd. Com. (China). The end-column ECL cell was composed of a three-electrode

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Fig. 1 Cyclic voltammograms for $\text{Ru}(\text{bpy})_3^{2+}$ and CPM in a PBS buffer solution (pH 8.0) at a bare Pt electrode. (a) Blank buffer solution only; (b) containing 5 mM $\text{Ru}(\text{bpy})_3^{2+}$; and (c) containing 5 mM $\text{Ru}(\text{bpy})_3^{2+}$ and 2 mM CPM. Scan rate, 50 mV/s.

system with a 300- μm diameter Pt disc electrode as a working electrode, a Pt wire electrode as an auxiliary electrode, and Ag/AgCl electrode as a reference electrode. A potentiostat was used to supply the potential of the oxidation of $\text{Ru}(\text{bpy})_3^{2+}$ to $\text{Ru}(\text{bpy})_3^{3+}$ at the working electrode. A piece of capillary (i.d. 25 μm , o.d. 375 μm), from Yongnian Optical Conductive Fiber Plant (Hebei, China), cut to 50 cm in length, was used as a separation capillary and was placed between the injection reservoir and the reaction reservoir. A high voltage was applied at the injection end, with the reservoir in the ECL detection cell held at the ground potential. During the experiments, the high-voltage power-supply not only provided a separation voltage across the capillary to drive sample, but also introduced the sample by electromigration injection at a definite time. The detection cell was placed directly above the photo-multiplier tube. The biased potential was set at 800 V. The sample was quantified using the ECL intensity. Before analysis, about 250 μL of the $\text{Ru}(\text{bpy})_3^{2+}$ solution (PBS, pH 8.0) was added into the reservoir, and refreshed every 2 h to eliminate the possible depletion effect or potential interference during the analysis. Before each run, the capillary was flushed with the corresponding running buffer until the baseline of CL was flat. All of these apparatus were controlled and data were collected with a programmable computer.

Binding of HSA with CPM

Equilibrium dialysis was applied to study the binding of HSA with CPM by incubating 150 μL of 200 μM HSA and 200 μL 0.01 M CPM in the dialysis bag at 37°C. The bag was then immersed in 4 mL of PBS, and 100 μL of outside solution was detected every half hour until the ECL intensity was constant. Then, the binding constant was also estimated by fitting to the Langmuir equation. The UV-visible spectra of the solution outside or inside of the dialysis bag were detected before or after culture to confirm the binding of HSA with CPM.

Results and Discussion

Cyclic voltammograms of $\text{Ru}(\text{bpy})_3^{2+}$ and CPM

Cyclic voltammograms were performed on a CHI 660 A electrochemical workstation (Shanghai Chenhua Apparatus, China) with a conventional three-electrode system. Pt wire encapsulated in epoxy resin was used as the working electrode, with an Ag/AgCl electrode as the reference electrode and a

Fig. 2 Influence of the applied potential on the ECL intensity.

platinum electrode as the auxiliary electrode. Figure 1 shows cyclic voltammograms of $\text{Ru}(\text{bpy})_3^{2+}$ in the presence or absence of CPM. Curve a shows no obvious peak in the cyclic voltammogram of a bare Pt electrode in a blank buffer solution (pH 8.0). In a buffer solution containing 5 mM $\text{Ru}(\text{bpy})_3^{2+}$, the cyclic voltammogram displays reversible electrochemical properties with a peak-to-peak separation of about 59 mV in continuous scanning (only one cycle shown here, curve b). In curve c, in a solution containing 5 mM $\text{Ru}(\text{bpy})_3^{2+}$ and 2 mM CPM, the reduction peak becomes weaker, while an obvious electrochemical catalytic oxidation peak can be discovered. Continuous cycling caused a continuous decrease of the oxidation peak (not shown). ECL is a means for converting electrical energy into radiative energy. At the electrode, the reactive intermediates were produced from stable precursors, and then to form excited states that can emit light. According to earlier reports,^{9,17} the mechanism can be illustrated as follows:

It was found that with the aid of CPM ($\text{RR}'\text{NCH}_2\text{R}''$), the quantity of $\text{Ru}(\text{bpy})_3^{2+}$ could be greatly enhanced while $\text{Ru}(\text{bpy})_3^{3+}$ decreased; therefore, the oxide current dramatically increased with a decrease in the reduce current.

Optimization of experimental conditions

Since the potential applied to the electrode had an effect on the rate of the light-emitting chemical reaction, it could influence the intensity of the emitted light. Therefore, the potentials of 1.0–1.4 V were investigated. In Fig. 2, it can be found that the ECL response increased if the potential increased from 1.0 to 1.2 V, and then decreased after 1.2 V. From Fig. 1, it can be found that the maximum oxide current for $\text{Ru}(\text{bpy})_3^{2+}$ -CPM system was at about 1.15 V, so the highest response may have been at about 1.2 V. However, when the potential was higher than 1.25 V, the oxidation of water may have had a negative effect on the ECL intensity. Therefore, in our following

Fig. 3 Effect of the injection time (A) and the injection voltage (B) on the ECL intensity (○) and the number of theoretical plates (N ; △) for 30 μ M CPM. The injection voltage was 10 kV in A and the injection time was 15 s in B. The separation voltage was 15 kV.

experiments, 1.2 V was set as the optimal detection potential.

The injection volume of the sample is another key experimental condition, since it can influence not only the sensitivity, but also the peak shape. Therefore, the effects of the injection voltage and the injection time on the ECL intensity were investigated in detail, which is shown in Fig. 3. With increasing the injection voltage over a range from 7 to 13 kV and increasing the injection time from 4 to 15 s, the ECL intensity increased, whereas the number of theoretical plates (N) decreased. From Eqs. (1)–(6), the CPM concentration is relative to the electrochemiluminescence reaction rate, which has an influence on the emission intensity; therefore, at a higher injection voltage, more analyte appears on the surface of the working electrode, which produces a higher ECL signal. However, too much analyte could not immediately react at the electrode, and was dispersed into the solution, so the ECL peaks were usually broadened and N decreased. Thus, to obtain a higher ECL signal and a larger N , 10 kV and 10 s was compromised as the optimal injection voltage and injection time, respectively.

The separation voltage was also investigated. With an increase in the separation voltage, the separation time decreased. However, no obvious variety in the ECL intensity was found. Considering a suitable separation time, 15 kV was selected as the separation voltage.

The pH of the solution is an important factor that influences the CL reaction rate and the resolution, because the $\text{Ru}(\text{bpy})_3^{2+}$ ECL reaction with alkylamine is a pH-dependent process, with the maximum emission being observed under a slightly basic condition.¹⁸ Therefore, the running buffer condition was tested from pH 4.5 to 9 prepared from PBS. As shown in Fig. 4, with an increase in the pH, the ECL response increases because of

Fig. 4 Effect of the buffer pH on the ECL intensity of $\text{Ru}(\text{bpy})_3^{2+}$.

Fig. 5 Typical ECL response for different concentrations of CPM. (a) 2 μ M, (b) 10 μ M, (c) 0.1 mM and (d) 0.5 mM.

less protonation of amines;¹⁹ however, above pH 8, the emitted light decreased, which may be ascribed to the reduced availability of $\text{Ru}(\text{bpy})_3^{3+}$ due to a competitive reaction with the OH^- ion. This pH-dependence might also be consistent with the earlier reports that showed maximum ECL activity of the tertiary amine occurs at a pH value lower than the pK_a of the amine.²⁰

Analytical performance

Therefore, the following experimental conditions were selected as the optimal conditions: applied potential, 1.2 V; injection voltage, 10 kV; injection time, 10 s; separation voltage, 15 kV; pH, 8.0. The typical ECL response curves for different concentrations of CPM are shown in Fig. 5. It can be seen that the ECL intensity increased and the peak was broadened as the concentration increased. It is reasonable that at larger concentrations, a larger amount of the analyte can react at the electrode to produce a higher intensity, whereas too much analyte may retard the reaction time. Therefore, in Fig. 6, the linear response curve of the ECL intensity with CPM concentrations, two linear response ranges can be found: at a higher CPM concentration, *i.e.*, between 15 μ M and 1 mM,

$$y = 595.2 + 7.1x \quad (r = 99.1\%); \quad (7)$$

while at a lower concentration, *i.e.*, from 0.8 μ M to 15 μ M (the inset of Fig. 6),

$$y = 95.9 + 35.6x \quad (r = 97.9\%). \quad (8)$$

Here y is the ECL intensity, and x is the CPM concentration in μ M. Comparing Eqs. (7) with (8), it can be found that the slope of Eq. (8) was much larger than that of Eq. (7), the reason of

Fig. 6 Linear response of the ECL intensities with the CPM concentrations. The inset is the CPM concentration from 0.8 to 15 μM .

which may be that at larger concentration, the quantity of the analyte is so high that CPM cannot immediately react at the electrode, and is scattered back into the buffer solution and diluted; however, at lower concentrations, the injected CPM can react completely when it just reaches the reservoir. The experimental detection limit, defined as the lowest concentration of CPM that gives rise to a signal-to-noise of 3, was found to be 0.5 μM .

Reproducibility

The repeatability of this experiment was investigated by four successive injections of 10 μM and 500 μM CPM, respectively. The interday variation was also studied. All of the results can be found in Table 1, which showed an excellent repeatability for CPM detection with CE-ECL.

Applications of CE-ECL detection for CPM

Tests of the recovery efficiencies for known amounts of CPM in urine were made. A series of sample solutions were prepared by adding certain amounts of CPM into 10 ml urine of healthy people, and then diluted to 50 ml with double-distilled water. The sample solution was then extracted with a syringe and injected into a 1 ml buffer solution for CPM detection. Table 2 gives the results and recoveries by the standard addition method. Actually, many other substances in urine also possess tertiary amine groups, and may cause an ECL response; however, the migration time at about 270 s was defined as for CPM, and the dilution effect was considered when calculating the founded CPM.

The association of HSA with CPM

In this work, equilibrium dialysis was adopted to study the binding of HSA with CPM by incubating 150 μL of 200 μM HSA and 200 μL 0.01 M CPM in the dialysis bag at 37°C. The solution outside of the bag was 4 mL PBS, and 100 μL of this solution was analyzed every half hour. To minimize the possible incidental error of the apparatus, the ECL intensity of 5×10^{-4} M CPM was detected and the relative ECL intensity was used to reflect the equilibrium process as follows:

$$\text{Relative ECL intensity} = \frac{\text{ECL intensity}_{\text{outside}}}{\text{ECL intensity}_{\text{total}}} \quad (9)$$

In Fig. 7, with an increase in time, the relative intensity increased, and reached a plateau (71%) after 4 h. After the equilibrium, 50 μL of the solution outside the dialysis bag (d), and 50 μL of the solution in the dialysis bag (e) were put into

Table 1 Reproducibility of successive and interday detection of CPM

CPM concentration/ μM	Migration time/s		ECL intensity/a.u.	
	Mean	RSD, %	Mean	RSD, %
10 ^a	270	0.42	450	2.48
500 ^a	273	0.94	4010	3.54
10 ^b	271	1.65	452	2.69
500 ^b	275	2.02	3998	4.22

a. The consecutive detection of four injections of CPM.

b. The interday detection of CPM.

Table 2 Determination of CPM determination in urine using CE-ECL

Added CPM/mg	Founded CPM ^a /mg	Recovery, %
48.8	47.8 $\pm$ 0.5	98.0
97.7	96.2 $\pm$ 1.0	98.5
146.5	143.0 $\pm$ 2.2	97.6
198.5	195.1 $\pm$ 3.0	98.5
244.2	241.3 $\pm$ 4.0	98.8

a. Values are mean $\pm$ S.D. ($n = 3$).

the 1.0 mL PBS buffer, respectively, and the corresponding UV-visible spectra were recorded (Fig. 8). For a comparison, 50 μL 10^{-2} M CPM (a), 20 μL 200 μM HSA (b), 50 μL 10^{-2} M CPM + 20 μL 200 μM HSA (c) were also injected into 1.0 mL PBS buffer, and UV-visible spectra were recorded again. Comparing curve d with curve a, the similarity of the peaks implied that only CPM could be found in the outside solution. Furthermore, it can be seen that the spectrum in the dialysis bag (e) is greatly different from the peak of HSA (b), CPM (a) and the simple associate spectrum of HSA with CPM without culture (c), which confirmed the binding of HSA and CPM under the experimental conditions.

Subsequently, the binding constant of HSA and CPM was estimated by the isotherm curve. In a series of dialysis bags, 50 μL 200 μM HSA and different volumes of 10^{-2} M CPM were mixed, and then immersed in 2 mL of the PBS buffer. The ECL intensity of the outside solution was detected after the binding was balanced (about 4 h). Figure 9 shows the relationship between the relative ECL intensity and the associated CPM with the injected CPM concentration. At the given HSA concentration, we could use the Langmuir isotherm equation,²¹

$$\theta = \frac{Kc}{1 + Kc}, \quad (10)$$

where θ is the binding coefficient, K , the binding constant, and c , the CPM concentration. Furthermore,

$$\theta = \frac{\alpha_c}{\alpha_{\max}}, \quad (11)$$

where α_c is the CPM binding amount (unit: mol) at different concentrations and $\alpha_{\max}$ is the maximum binding amount under this condition. Therefore, by combining of Eqs. (10) and (11), the following equation can be obtained:

$$\alpha_c = \frac{Kc\alpha_{\max}}{1 + Kc}. \quad (12)$$

Fig. 7 Relationship of the relative ECL intensity with the reaction time.

The bound CPM in Fig. 9 can be non-linearly fitted using the above equation, and expressed as

$$\alpha_c = 2.24 \times 0.41c / (1 + 0.41c) \quad (q_r = 2.14 \times 10^{-4}). \quad (14)$$

Considering the units in the plot, for the HSA used in this section, the maximum binding amount is 2.24×10^{-4} M, and the binding constant is 4.1×10^3 M⁻¹.

Conclusions

In this work, CPM, because of its tertiary amine group, was firstly detected using the CE-Ru(bpy)₃²⁺ ECL technique as a simple, fast and selective method. The optimal analytical conditions, the reproducibility and the linearity were considered in detail. Two linear responses to CPM, *i.e.*, from 15 μM and 1 mM and from 0.8 μM to 15 μM, were obtained with a detection limit of 0.5 μM. The binding of CPM with HSA was estimated to be 4.1×10^3 M⁻¹ by CE-Ru(bpy)₃²⁺ ECL. Therefore, CE-Ru(bpy)₃²⁺ ECL is a well-established, sensitive detection technique for the determination of amine-containing pharmaceuticals, purines and DNA without any derivation.

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Fig. 8 UV-visible spectra of 50 μL 10⁻² M CPM (a), 20 μL 200 μM HSA (b), 50 μL 10⁻² M + 20 μL 200 μM HSA (c), 50 μL of the solution outside the dialysis bag (d), and 50 μL of the solution in the dialysis bag (e). Inset A is an enlargement of the right figure from the 240 nm to 310 nm.

Fig. 9 Relationship of the relative ECL intensity outside the dialysis bag and the bound CPM on the CPM concentration.

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