

Electrochemical determination of hydrogen peroxide using *o*-dianisidine as substrate and hemoglobin as catalyst

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Abstract. A new electrochemical method for the determination of microamounts of hydrogen peroxide utilizing *o*-dianisidine (ODA) as substrate and hemoglobin (Hb) as catalyst is described in this paper. Hb can be used as mimetic peroxidase and it can catalyse the reduction of hydrogen peroxide with the subsequent oxidation of ODA. The oxidative reaction product is an azo compound, which is an electroactive substance and has a sensitive second-order derivative polarographic reductive peak at the potential of -0.58 V (vs. SCE) in pH 8.0 Britton–Robinson (B–R) buffer solution. The conditions of Hb-catalytic reaction and polarographic detection of the reaction product were carefully studied. By using this polarographic peak and under optimal conditions, the calibration curve for the H_2O_2 was constructed in the linear range of 2.0×10^{-7} ~ 1.0×10^{-4} mol/l with the detection limit of 5.0×10^{-8} mol/l. This method can also be used to the determination of Hb content in the range of 2.0×10^{-9} ~ 3.0×10^{-7} mol/l with a detection limit of 1.0×10^{-9} mol/l. The proposed method was further applied to the determination of the content of H_2O_2 in fresh rainwater with satisfactory results. The catalytic reaction mechanism and the electrode reductive process of the reaction product were carefully studied.

Keywords. Hemoglobin; hydrogen peroxide; *o*-dianisidine, polarography.

1. Introduction

Hemoglobin (Hb) is an important respiratory protein in red cells of human blood, which is used as the carrier of oxygen. Its concentration in blood is associated with some clinical diseases such as heart disease, anemia, etc. Similarly, hydrogen peroxide plays an important role in the clinical and biological fields. The oxidation of many biological substances in body fluids produces a certain amount of hydrogen peroxide.^{1,2} Further, the content of hydrogen peroxide in the environment is important because it is a key species in the reaction of the troposphere.³ So the sensitive determination of both Hb and H_2O_2 has aroused great interest in analytical chemistry.

Until now many methods have been proposed for the analysis of H_2O_2 or Hb. For the determination of H_2O_2 , methods such as electroanalysis,⁴ spectrophotometry,⁵ fluorescence⁶ etc. have been established. For the determination of Hb, the commonly used method is spectrophotometry.^{7–9} However, the spectrophoto-

metric detection is less sensitive due to the greater influence of hemolysis and turbidity of biological sample. Electrochemical analysis has inherent advantages over spectrophotometry, such as lower detection limit and wide dynamic range. The determination of Hb with electrochemical methods has been reported,^{10,11} but most of them focused on the chemically modified electrode with some redox mediators such as methylene blue, which can enhance the charge transfer rate of Hb on the electrode surface. But those methods are handicapped by the difficult preparation and poor reproducibility of modified electrode.

Hb consists of four polypeptide subunits and an iron-porphyrin heme as the active centre. So it has the inherent reactivity as a peroxidase, which can catalyse the reduction of hydrogen peroxide and the oxidation of a hydrogen donor substrate at the same time. Based on this principle, Hb has been used as the mimetic enzyme of peroxidase. Zhang *et al*¹² reported a new stopped flow spectrophotometric method for the determination of Hb, based on its enzymatic activity using *o*-phenylenediamine as substrate. Wang¹³ applied Hb as the peroxidase substitute in the catalytic oxida-

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tion of *p*-cresol by H_2O_2 and further established a sensitive spectrofluorimetric method for the determination of trace H_2O_2 .

In this paper, a new electrochemical method for the determination of trace amount of H_2O_2 and Hb using *o*-dianisidine (ODA) as substrate is reported for the first time. ODA has been used as the chromogen substrate for HRP and HRP-based enzyme immunoassay.^{14,15} So in this paper ODA was selected as the electrochemical substrate of Hb catalytic reaction for the determination of H_2O_2 . The conditions for Hb- H_2O_2 -ODA reaction system were carefully optimized and the electrochemical behaviour of oxidative reaction product was investigated. Based on the electrochemical reduction of the reaction product on the mercury electrode, a sensitive electrochemical method for analysis of H_2O_2 and Hb is established, which is successfully attempted to determine H_2O_2 content in fresh rainwater with satisfactory results.

2. Experimental

2.1 Apparatus

A model JP-303 polarographic analyzer (Chengdu Instrument Factory, China) was used for all polarographic detections with the traditional three electrodes system involving a dropping mercury as working electrode, a platinum wire auxiliary electrode and an saturated calomel reference electrode (SCE). All the cyclic voltammetric experiments were carried out using a DS-2004 electrochemical analyser (Shandong Dongsheng Electronic Instrument, China) with a DS-991 static mercury drop electrode (Shandong Dongsheng Electronic Instrument, China) as working electrode, a saturated calomel reference electrode (SCE) and a platinum wire auxiliary electrode. The UV-Vis absorption spectrum was measured on a Cary 50 probe UV-Vis spectrophotometer (Australia Varian Company). A thermostat bath was used to control the reaction temperature.

2.2 Materials

Hemoglobin (bovine, Hb, Biochemical grade) was purchased from Tianjin Chuanbei Bio-product Company with the molecular weight of 64500, 1.0×10^{-4} mol/l stock solution was prepared and stored at 4°C. The working solution of Hb was prepared daily by diluting the stock solution to the suitable concentration. 1.0×10^{-2} mol/l *o*-dianisidine (ODA, Sigma) solution in

methanol was prepared and stored in the dark. H_2O_2 solution was prepared by appropriate dilution of commercial 30% H_2O_2 with water and standardized by titration with KMnO_4 . 0.2 mol/l Britton-Robinson (B-R) buffer solution (pH 8.0) and 0.1 mol/l KH_2PO_4 - Na_2HPO_4 buffer solution (pH 6.6) were prepared respectively to control the pH of reaction solution. All chemicals used are of analytical reagent grade and doubly distilled water was used for preparing the solutions.

2.3 Procedure

In a 10 ml colorimetric tube, 0.5 ml pH 6.6 KH_2PO_4 - Na_2HPO_4 buffer solution and 2.0 ml of 1.0×10^{-2} mol/l ODA was mixed with a suitable amount of H_2O_2 and Hb solutions in sequence, then diluted to 10 ml with methanol. After reaction at 50°C for 50 min, the reaction solution was cooled with ice-water to stop the catalytic reaction. Then 5.0 ml of reaction solution was transferred to another 10 ml colorimetric tube and 1.5 ml pH 8.0 B-R buffer solution was added, diluted to the scale and shaken to mix homogeneously. The second order derivative linear-sweep polarographic peak of the reaction product was recorded and the reductive peak current at -0.58 V (vs. SCE) was measured.

3. Results and discussion

3.1 UV-Vis absorption spectra

Figure 1 shows the UV-Vis absorption spectra of Hb- H_2O_2 -ODA catalytic system in the wavelength range from 300 to 700 nm. Curve 1 is the mixture of ODA with H_2O_2 and it shows no absorbance. After adding the different amount of Hb solution and reacted at 50°C for 50 min, a new absorption peak appears at 438 nm and increases with the addition of the amount of Hb, which indicated a new reaction product was formed due to the Hb catalysed H_2O_2 oxidation of ODA.

3.2 Typical second-order derivative linear sweep polarogram of Hb- H_2O_2 -ODA catalytic system

As shown in figure 2, the typical second order derivative linear sweep polarogram of Hb- H_2O_2 -ODA catalytic system was recorded. Curve 1 is the polarogram of buffer solution, which has no polarographic peak. Curve 2 is the polarogram of ODA + Hb solution,

it has a small blank wave, which is due to the oxidation of ODA by air. Curve 3 is the polarogram of ODA + H_2O_2 solution, which is similar as curve 2 and is due to the slowly oxidation of ODA by H_2O_2 . Curve 4 is the mixture of ODA + Hb + H_2O_2 , which has a well-defined polarographic reductive peak at the potential of -0.58 V (vs SCE). Due to the addition of H_2O_2 and Hb, the Hb-catalytical oxidation reaction is speeded up and a new electroactive reaction product is formed. The more the H_2O_2 or Hb added, the more the oxidation product is generated and the higher the peak current is got. The peak current is in proportion to the concentration of H_2O_2 and Hb, so this method can be applied to the determination of H_2O_2 or Hb content in real samples.

3.3 Electrochemical behaviour of Hb catalytic reaction product

In our previous paper,¹⁶ the oxidative reaction of horseradish peroxidase(HRP) catalysed H_2O_2 with ODA have been studied and reported. Because of the similar catalytical function of HRP with Hb, so the reaction of ODA and H_2O_2 with Hb can also produce a new azo-compound though the reaction scheme shown in figure 3. This bisazobiphenyl product is produced from the condensation of two molecules of the *o*-dianiside quinonediimine, which can easily be reduced on the mercury electrode. Thus the catalytic reaction can be monitored by polarographic detection of the reductive current, which was generated

by reductive reaction of the azo group of the product. So the electrochemical properties of this bisazo product were carefully studied.

For the Hb catalytic reaction solution and under the selected conditions, the cyclic voltammogram of this bisazo product was recorded and is shown in figure 4. It can be seen that the product had a pair of redox peaks with the anodic peak potential (E_{pa}) as -0.44 V and the cathodic peak potential (E_{pc}) as -0.59 V. So the apparent formal potential (E^0) has been calculated as -0.515 V, since E^0 is the average of E_{pa} and E_{pc} . The peak potential separation $\Delta E_p = |E_{pa} - E_{pc}|$ is calculated as 0.15 V with the peak current of $I_{pa}/I_{pc} \neq 1$, which indicates that the electrode process of the oxidative product on the mercury electrode was quasi-reversible. The reductive peak currents increase with the scanning rate in the range of $100 \sim 800$ mV/s. The plot of peak current with square root of the scanning rate is not linear but an upward curve, which indicates that the azo product can be absorbed on the surface of mercury electrode. The peak current also increases with the increasing of the static period of mercury drop. In multiple sweep cyclic voltammograms the peak current decreases gradually with increasing scan times. All this electrochemical experiments results show that the reaction product can be strongly absorbed on to the mercury electrode. The plot of the peak potential against the pH of buffer is a straight line with a slope of -0.058 in the pH range of $6.0 \sim 10.0$. According to the equation, $-0.059x/n = -0.058$, where

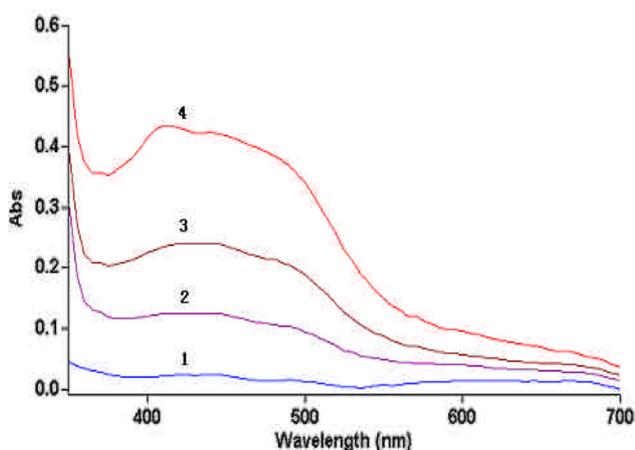


Figure 1. UV-Vis absorption spectra of Hb- H_2O_2 -ODA catalytic system in the absence and present of different amount of Hb. (1) 1.0×10^{-3} mol/l ODA + 2.0×10^{-3} mol/l H_2O_2 ; (2) (1) + 1.0×10^{-5} mol/l Hb; (3) (2) + 2.0×10^{-5} mol/l Hb; (4) (3) + 5.0×10^{-5} mol/l Hb.

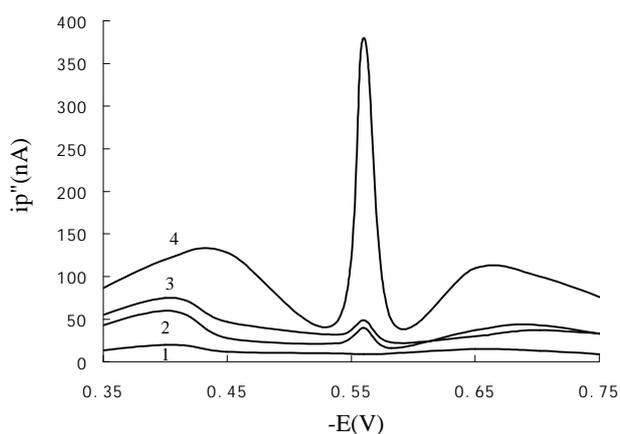


Figure 2. The linear sweep second order derivative polarogram of Hb- H_2O_2 -ODA catalytic system. (1) 0.05 mol/l KH_2PO_4 - Na_2HPO_4 buffer solution (pH 6.6); (2) (1) + 1.0×10^{-3} mol/l ODA + 1.0×10^{-7} mol/l Hb; (3) (1) + 1.0×10^{-3} mol/l ODA + 1.5×10^{-5} mol/l H_2O_2 ; (4) (2) + 1.5×10^{-5} mol/l H_2O_2 .

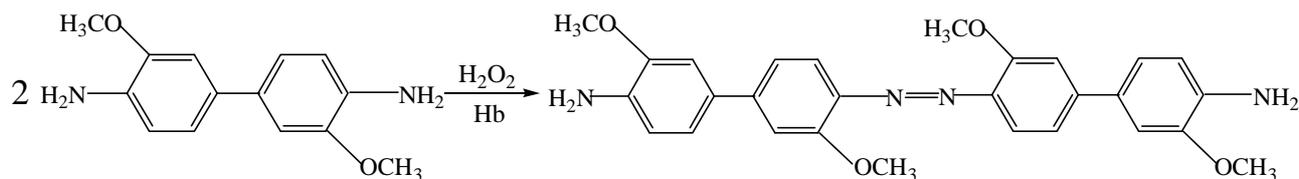


Figure 3. Catalytic oxidation reaction scheme.

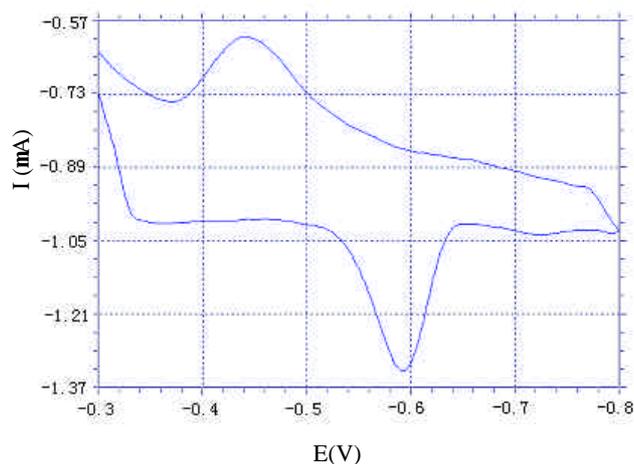


Figure 4. Cyclic voltammogram of the oxidation product under selected conditions with the scan rate as 300 mV/s.

n is the electron transfer number and x is the hydrogen ion number participating in the reaction, so $x = 2$ and $n = 2$. From the above experimental results, the azo product appears to undergo two-electron adsorptive quasi-reversible reduction on the Hg electrode as shown in figure 5.

3.4 Optimization of the conditions of Hb catalytic reaction and polarographic detection

3.4a Effect of pH and buffers: The kind of the buffer solution and pH of the reaction buffer greatly affects the activity of Hb. Differential buffer solution such as phosphate, B-R, carbonate, etc. were tested and the results show that in KH_2PO_4 - Na_2HPO_4 buffer solution the activity of Hb is the highest. Figure 6 shows the dependence of pH with peak currents in the range from 5.0 to 7.5. The peak current reaches maximum in the pH range of 6.2 ~ 6.6, so pH 6.6 was recommended for use in our work and the addition of 0.5 ml of 0.1 mol/l phosphate buffer solution in 10 ml final solution is chosen.

3.4b Reaction time and temperature: Under the above conditions, the influence of temperature on the Hb catalytic reaction was examined with the reaction temperature in the range from 20 to 70°C and the results are shown in figure 7. When the temperature is lower than 50°C, the peak current of the catalytic product increases with the increase of temperature and it reaches a maximum at 50°C; when the temperature exceeds 50°C, the peak current decreases correspondingly, which may be due to the denaturing of protein at high temperature. Therefore, a reaction temperature of 50°C is suggested for the catalytic reaction. At this temperature, the peak current reaches the maximum for 50 min and remains constant for at least 3 h.

3.4c Optimal concentration of the substrate: ODA is an excellent spectrophotometric and electrochemical substrate in HRP- H_2O_2 catalytic system, so it was chosen as the substrate for Hb. Results show that the peak current increases with the increasing of the concentration of ODA up to 2.0×10^{-3} mol/l and then decreases thereafter. So a concentration of ODA is chosen as 2.0×10^{-3} mol/l.

3.4d Optimal conditions for polarographic detection: Polarographic behaviour of the Hb-catalytic reaction product is greatly influenced by the acidity of the supporting electrolyte. After the catalytic reaction, the effect of the pH of the supporting electrolyte on the electrochemical response of the enzymatic product was checked in the pH range from 6.0 to 10.0. The results show that in pH 8.0 B-R buffer supporting electrolyte solution, peak current reaches the maximum and decreases with further increase of the buffer pH (Figure 8). Under the selected conditions, the polarographic peak current remains stable for about 2 h.

The optimal instrumental conditions for the detection were selected as follows: initial potential: -0.40 V; mercury drop standing time: 13 s and potential scanning rate: 1000 mV/s.

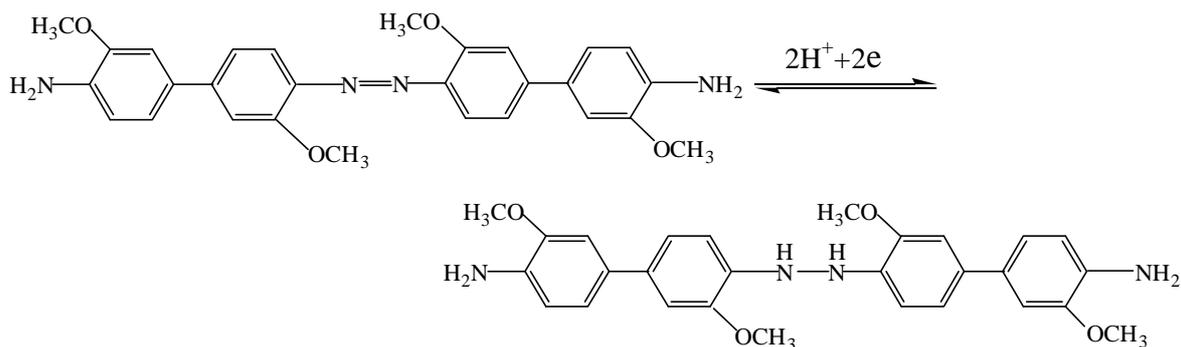


Figure 5. Electro-reduction scheme.

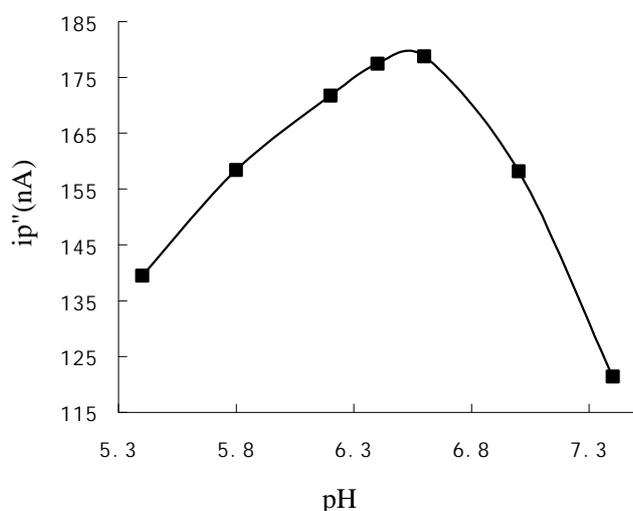


Figure 6. Influence of pH on the catalytic reaction. Concentrations of ODA, H_2O_2 and Hb are 1.0×10^{-3} mol/l, 4.0×10^{-6} mol/l, 1.0×10^{-7} mol/l. Phosphate buffer solutions of different pH values are used.

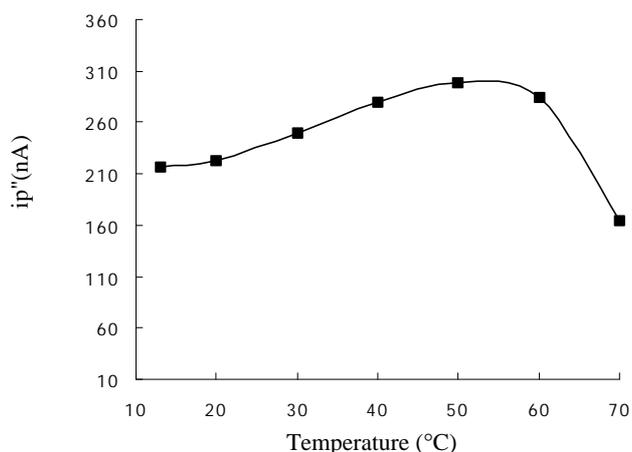


Figure 7. The influence of temperature on catalytic reaction. Concentrations of ODA, H_2O_2 and Hb are 1.0×10^{-3} mol/l, 1.0×10^{-5} mol/l, 1.0×10^{-7} mol/l with pH 6.6 phosphate buffer solution.

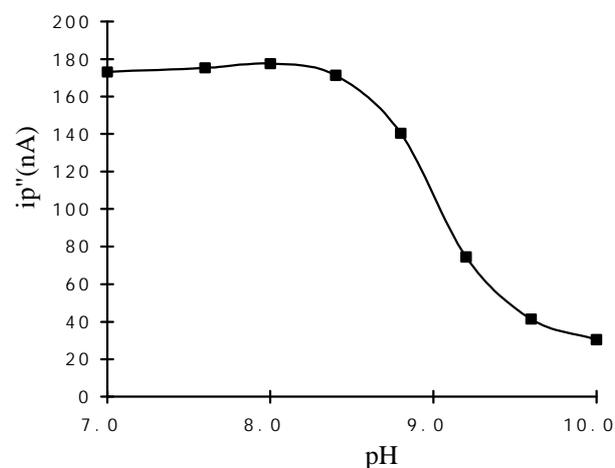


Figure 8. The influence of pH on the peak current. Concentrations of ODA, H_2O_2 and Hb are 1.0×10^{-3} mol/l, 4.0×10^{-6} mol/l, 1.0×10^{-7} mol/l.

3.5 Analytical application

3.5a *Quantitative determination of H_2O_2* : Under optimal reaction and detection conditions, the calibration curve of peak current versus the concentration of H_2O_2 was constructed with 2.0×10^{-3} mol/l ODA and 1.0×10^{-7} mol/l Hb. Good linearity can be found as follows: (1) in the range of 2.0×10^{-7} to 1.0×10^{-5} mol/l with the linear regression equation as ip'' (nA) = $40.30 + 29.30C$ (μ mol/l), ($g = 0.998$, $n = 13$); (2) in the range of 1.0×10^{-5} to 1.0×10^{-4} mol/l with the linear regression equation as ip'' (nA) = $248.08 + 8.47C$ (μ mol/l), ($g = 0.997$, $n = 6$), where ip'' (nA) is the reductive peak current and C is the concentration of H_2O_2 . The relative standard deviation for 11 parallel determinations of 1.0×10^{-7} mol/l H_2O_2 was 3.01% and the detection limit was 5.0×10^{-8} mol/l.

3.5b *Sample determinations of H_2O_2 and recovery test*: Under the recommended conditions, the proposed method was applied to determination of the

Table 1. Analytical results of the content of H₂O₂ in fresh rainwater.

Sample	Found ($\times 10^{-5}$ mol/l)			Average ($\times 10^{-5}$ mol/l)	RSD (%)	Added ($\times 10^{-5}$ mol/l)	Detected ($\times 10^{-5}$ mol/l)	Recovery (%)
1	6.59	6.72	7.17	6.86	3.91	10.00	16.78	99.18
	6.66	7.22	6.81					
2	3.28	3.36	3.59	3.43	4.01	10.00	13.78	103.52
	3.33	3.61	3.41					
3	5.44	5.36	5.87	5.57	3.35	10.00	15.45	98.79
	5.47	5.61	5.69					

content of H₂O₂ in fresh rainwater. The analytical results are listed in table 1. The recovery tests show that this method is practicable and reliable.

3.5c Calibration curve for Hb assay: In this system Hb was used as the catalyst and the concentration of Hb greatly influenced the catalytic reaction. On adding more Hb, the peak current increased. Hence, this new electrochemical method can also be used for Hb detection. Under the selected Hb-catalytic reaction and detection conditions with 2.0×10^{-3} mol/l ODA and 4.0×10^{-3} mol/l H₂O₂, the second-order derivative linear sweep polarographic peak current also has a good linear relationship with the concentration of free Hb in the range from 2.0×10^{-9} to 3.0×10^{-7} mol/l and the linear regression equation is ip'' (nA) = $14.32 + 3.44C$ (nmol/l), ($g=0.997$, $n=16$), where ip'' (nA) is the reductive peak current and C is the concentration of Hb. The relative standard for 11 parallel determination of 2.0×10^{-9} mol/l Hb was 2.95% and the detection limit was 1.0×10^{-9} mol/l.

4. Conclusion

In this work Hb was used to catalyse the reduction of hydrogen peroxide, with the subsequent oxidation of *o*-dianisidine (ODA) and the reaction product was reduced at the mercury electrode. Based on the reductive current of the Hb-catalysed reaction product, a new electrochemical method for the determination of microamount of hydrogen peroxide was established and further applied to the determination of the content of H₂O₂ in fresh rainwater with satisfactory results.

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