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The Influencing of Preanodized Inlaying Ultrathin Carbon Paste Electrode on the Oxidation for the Xanthine and Hypoxanthine by the Hydrogen Bond

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In this paper, a pre-anodized inlaying ultrathin carbon paste electrode (PAIUCPE) with 316L as a matrix was constructed by a simple and fast electrochemical pretreatment. Using xanthine (Xa) and hypoxanthine (HXa) as the target compounds, the pH effects compositions of buffer solution, the accumulation times, hydrogen bond catalysis, degree of auxiliary electrode reaction on the size of peak currents (I_p) of Xa and HXa was discussed in detail. Also, it was proposed that Xa and HXa were respectively absorbed at the surface of PAIUCPE through hydrogen bonding. The influencing mechanisms of the PAIUCEP on electrochemical oxidation of Xa and HXa were explained in detail. Moreover, the linear relationships for the Xa and HXa were obtained in the range of 6×10^{-8}-3×10^{-5} mol/L and 2×10^{-7}-7×10^{-5} mol/L, respectively. The detection limits for the Xa and HXa were 1.2×10^{-8} mol/L and 5.7×10^{-8} mol/L, respectively. Moreover, this proposed method could be applied to determine the Xa and HXa in human urine simultaneously with satisfactory results.

Keywords: Xanthine; Hypoxanthine; Hydrogen bond.

INTRODUCTION

Both xanthine (Xa) and hypoxanthine (HXa) are intermediates of purine metabolism and then form the final oxidation product of uric acid (UA). Purine compounds can influence the activities of the nervous system and have many physiological functions of sedation, such as dilating blood vessels and lowering blood pressure. The concentration levels of Xa and HXa in body fluids are sensitive indexes for the pathology conditions of gout, hyperuricemia and renal failure.[1-2] Therefore, simultaneous detection of these two compounds have considerable significance in biochemical and clinical diagnosis. Various methods have been reported to determine the purine degradation products, including high performance liquid chromatography (HPLC),[3] photoion mass spectroscopy,[4] chemiluminescence,[5] enzymatic method,[6-7] capillary electrophoresis (CE)[8] and electrochemistry,[2,9-13] whereas HPLC methods require fastidious sample preparations, prolonged analysis time and expensive materials, enzymatic methods are unstable and very expensive, and CE methods need expensive apparatus, only electrochemical approaches are relatively easy and fast for determination of Xa and HXa.

As we all know, no matter what the working electrode is employed in the electroanalytical chemistry, the acidity of the solution that has an important influence on the size of peak current and peak potential is always optimized as one of the preferential research conditions in general. In fact, so far it has not been reported in detail to the mechanism about the effect of acidity on the size of peak current in the current literature, and it is only simply reported about the concentration determinations for the Xa and HXa under a certain pH value,[2,9-13] especially, the mechanism that how the size of peak current is affected by the pH value has not been theoretically explained in detail. For the electrode reactions of giving up proton,[2,9-17] their oxidation potentials always shift negatively with the increase in pH, this indicates that the ability of electroactive groups to be oxidized should strengthen with the increase in pH. Accordingly, the oxidation current also should enlarge. As a matter of fact, some of their oxidation peak currents decrease with the increment of pH value all the while, and some other increase at first, then once the pH surpasses a certain value, the currents would once more reduce with the increase in pH value. Therefore, figuring out the effect of pH on the size of peak current is of an important significance to explore the electrode reaction mechanism.

It has been reported in the literatures[18-19] that all carbon surfaces are nearly prone to react with oxygen and water, and many oxygen-containing functional groups such as carboxyl (-COOH), phenolic hydroxyl (-OH), lactones, ethers (-O-) and some active sites would be generated by means of special electrochemical pretreatment (also called

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pre-anodized) for carbon paste electrode (CPE) on carbon surface. Moreover, the carbon surface can be disrupted significantly so as to form a film of "electrogenerated graphitic oxide (EGO)", which make it exhibit excellent electrocatalytic activity towards adsorption and electron transfer kinetics. However, the resistance of CPE usually not only varies with the changes of preparation conditions, but also analytes are easier to diffuse toward the interior of carbon paste electrode, so that its reproducibility would be affected.[20]

In order to minimize the diffusion extent of analytes from electrode surface to its interior and achieve good reproducibility, an inlaying ultrathin (about thickness of 100 nm) carbon paste electrode that adopted nichrome as a substrate is developed by Wang *et al.*,[21] which is easier to make, low cost and high stability. In this paper, a pre-anodized inlaying ultrathin carbon paste electrode (PAIUCPE) is constructed using the industry leftover 316L stainless that have commendable biocompatibility, oxidation resistance and corrosion resistance in the biological environment[22] as a matrix. The PAIUCPE has excellent electrochemical response to the Xa and HXa. In order to research the interaction mechanism between analytes (Xa, HXa) and PAIUCPE, in this paper, it is proposed that the Xa and HXa could be respectively absorbed on the PAIUCPE surface through hydrogen bonding. Comparing with inlaying ultrathin carbon paste electrode (IUCPE), the PAIUCPE could not only obviously decrease overpotentials of oxidation for Xa and HXa, but also promote markedly their oxidation peak currents. The reason may be substantially that the bond energy of carbon-hydrogen (C-H) bond of the electroactive groups for Xa and HXa are greatly weaken due to hydrogen bonding between analytes (Xa, HXa) and PAIUCPE surface. This shows that the hydrogen bond plays an important role in depressing the activation energy (*i.e.* overpotential) of electrode reaction. In brief, this paper focuses on the influencing mechanisms of the PAIUCEP on electrochemical oxidation of Xa and HXa, as well as the simultaneous determination of the Xa and HXa in human urine. Compared to the electrodes used in literatures,[9-14] this electrode is simple to prepare, low cost and avoids complex modification. Therefore, it is easier to popularize in conventional laboratory, and has a wide application prospect in the future.

RESULTS AND DISCUSSION

The interaction mechanism of PAIUCPE with $K_4[Fe(CN)_6]$

The cyclic voltammograms of 1.0 mmol/L $K_4[Fe(CN)_6]$/0.10 mol/L H_2SO_4 solution on different electrodes are shown in Fig. 1. A pair of oxidation–reduction peaks appeares at +0.491 V and +0.268 V at the IUCPE (*curve a*), and another pair of oxidation–reduction peaks appears at +0.379 V and +0.315 V at the PAIUCPE (*curve b*), and their peak separations are 223 mV and 64 mV at the IUCPE and PAIUCPE, respectively. Especially, the peak current at the PAIUCPE is markedly greater than that of the IUCPE, moreover the ratio of redox peak currents is approximately 1, the peak separation of 64 mV is also close to the ideal value of 58 mV which indicates that a reversible electron transfer process appears at the PAIUCPE, and the PAIUCPE could availably improve the electron transfer rate of electrochemical reaction. The reasons may be that negatively charged oxygen-containing functional groups such as carboxyl (-COOH), phenolic hydroxyl (-OH), ether(-O-) and ester, which could be generated on the surface of PAIUCPE during the process of pre-anodization, have excellent electrocatalytic activity towards adsorption and electron transfer kinetics.

By compared with the redox peak potential at the IUCPE, the oxidation peak potential shifts negatively 112 mV and the reduction peak potential shifts positively 47 mV at the PAIUCPE, but the reasons that why occurs a negative shift of the oxidation peak and a positive shift of the reduction peak have not been reported in current literature. Because the lone pair electrons on the oxygen-containing functional groups of PAIUCEP surface could be

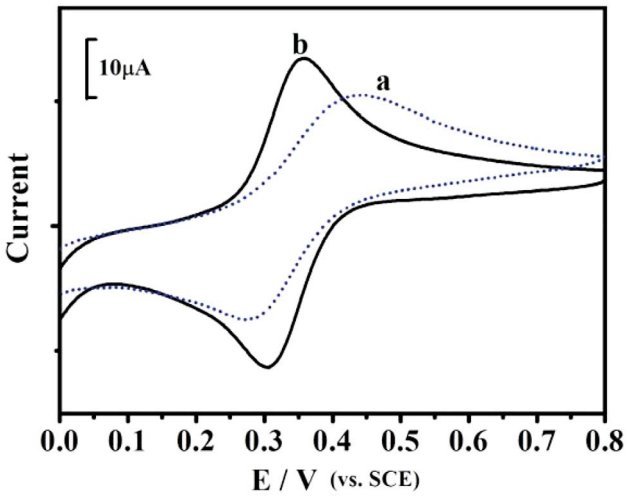


Fig. 1. Cyclic voltammograms of $K_4[Fe(CN)_6]$ on different electrodes. a-IUCPE, b-PAIUCPE; $C(K_4[Fe(CN)_6]) = 1.0 \times 10^{-3}$ mol/L; supporting electrolyte: 0.10 mol/L H_2SO_4;scan rate: 100 mV/s.

protonated in 0.10 mol/L of H_2SO_4, and a positively-charged(H^+) "Hemholtz" layer is formed at the surface of PAIUCEP. In consequence, the $[Fe(CN)_6]^{4-}$ and $[Fe(CN)_6]^{3-}$ are both adsorbed at the surface of PAIUCEP through electrostatic(or hydrogen bond) interaction in the ion association complexes $H^+\cdot[Fe(CN)_6]^{4-}$ and $H^+\cdot[Fe(CN)_6]^{3-}$. And their ion association reactions can be expressed as follows:

$$H^+ + Fe(CN)_6^{4-} \rightleftharpoons H^+\cdot Fe(CN)_6^{4-}$$
$$[H^+]\cdot[Fe(CN)_6^{4-}]\cdot K_{H+\cdot[Fe(CN)6]4-} = [H^+\cdot Fe(CN)_6^{4-}]$$
$$H^+ + Fe(CN)_6^{3-} \rightleftharpoons H^+\cdot Fe(CN)_6^{3-}$$
$$[H^+]\cdot[Fe(CN)_6^{3-}]\cdot K_{H+[Fe(CN)6]3-} = [H^+\cdot Fe(CN)_6^{3-}]$$

$K_{H+[Fe(CN)6]4-}$ and $K_{H+[Fe(CN)6]3-}$ are separately the ion association constants of $H^+\cdot Fe(CN)_6^{4-}$ and $H^+\cdot Fe(CN)_6^{3-}$. Based on the above discussion, the Nernst equation can be expressed as follows:

$$E = E° + 0.059log([H^+\cdot Fe(CN)_6^{3-}]/[H^+\cdot Fe(CN)_6^{4-}])$$
$$= E° + 0.059log([H^+]\cdot[Fe(CN)_6^{3-}]\cdot K_{H+\cdot[Fe(CN)6]3-}/[H^+]\cdot[Fe(CN)_6^{4-}]\cdot K_{H+[Fe(CN)6]4-})$$
$$= E° + 0.059log(K_{H+\cdot[Fe(CN)6]3-}/K_{H+[Fe(CN)6]4-}) + 0.059log([Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-})$$

Based on the ratio of redox peak currents for $Fe(CN)_6^{4-}$ and $Fe(CN)_6^{3-}$ is approximately 1, indicating that $[Fe(CN)_6]^{3-}$ and $[Fe(CN)_6]^{4-}$ or $[H^+\cdot Fe(CN)_6^{3-}]$ and $[H^+\cdot Fe(CN)_6^{4-}]$ at the PAIUCPE surface have same concentrations. Therefore, a positive or negative shift of the redox peak potential is related to the sizes of $K_{H+\cdot[Fe(CN)6]4-}$ and $K_{H+\cdot[Fe(CN)6]3-}$. As seen from the above formula, the greater the ion association constant (K), the more stable the ion association complex. Accordingly, the greater the degree of negative shift of oxidation peak potential and the degree of positive shift of reduction peak potential. The oxidation peak potential shifts negatively 112 mV and the reduction peak potential shifts positively 47 mV, indicating that the $K_{H+\cdot[Fe(CN)6]4-}$ of $[H^+\cdot Fe(CN)_6^{4-}]$ is greater than $K_{H+\cdot[Fe(CN)6]3-}$ of $[H^+\cdot Fe(CN)_6^{3-}]$. The reason may be that the negative charge of $Fe(CN)_6^{4-}$ is more high than that of $Fe(CN)_6^{3-}$, and the greater the number of charge, the stronger the electrostatic interaction. As a result, the ion association complex of $H^+\cdot Fe(CN)_6^{4-}$ which is formed between the $Fe(CN)_6^{4-}$ and H^+ in the "Hemholtz" layer is more stable than the $H^+\cdot Fe(CN)_6^{3-}$, and then the oxidation potential shifted negatively degree is greater than the reduction potential shifted positively degree. According to the redox peak currents for $Fe(CN)_6^{4-}$ and $Fe(CN)_6^{3-}$ being equal, indicating that $Fe(CN)_6^{3-}$ formed by $Fe(CN)_6^{4-}$ to be oxidized can be associated with H^+ in the "Hemholtz" layer by the electrostatic interaction *or* hydrogen bond, and make $Fe(CN)_6^{3-}$ change into $H^+\cdot Fe(CN)_6^{3-}$ absorbed at the PAIUCPE surface. Consequently, diffusing of $Fe(CN)_6^{3-}$ from the PAIUCPE surface toward bulk solution could be restrained, so that the size of reduction peak current is almost the same as the that of oxidation peak current.

The influencing mechanism of different supporting electrolytes on peak currents

The sizes of oxidation peak currents for Xa (1.0×10^{-5} mol/L) and HXa (1.0×10^{-5} mol/L) in buffer solutions containing different components at the PAIUCPE are measured. Compared with CH_3COOH-CH_3COONa and PBS solutions, the peak currents of Xa and HXa in B.R solution are the least. The reason may be that the empty orbits of the boric acid molecules in the B.R solution can accept the lone pair electrons of oxygen-containing functional groups at the PAIUCPE surface, thus form the "Helmhotz" layer with the adsorbing boric acid molecules, which weakens the interaction between the electrode surface and analytes (Xa, HXa), the concentrations of the analytes on the PAIUCPE surface reduces, leading to their peak currents becoming the least accordingly. In addition, the peak currents of Xa and HXa in CH_3COOH-CH_3COONa solution are smaller than the peak currents in PBS solution, the reasons may be as follows: 1) Because the protonation constant ($\beta^H = 1.3 \times 10^2$) of $H_2PO_4^-$ (the main components of PBS solution are $H_2PO_4^-$ and HPO_4^{2-}) was smaller than protonation constant ($\beta^H = 5.6 \times 10^4$) of CH_3COO^-, the ability of interaction between $H_2PO_4^-$ and electrode surface (for example, hydroxyl) through hydrogen bonding is lower than that of CH_3COO^-. 2) Although the protonation constant ($\beta^H = 4.0 \times 10^6$) of HPO_4^{2-} is greater than that of CH_3COO^-, the concentration (0.038 mol·L^{-1}) of HPO_4^{2-} is smaller than that (0.10 mol·L^{-1}) of CH_3COO^- at pH = 7.00. 3) Based on the above discussion, in comparison with the CH_3COOH-CH_3COONa and B.R solutions, the ability of interaction between PBS solution and electrode surface should be the least through chemical bond. So, PBS solution have a less influence on the interaction between the electrode surface and the analytes, the peak currents of Xa and HXa obtain their maxima, respectively. Finally, the PBS is selected as the supporting electrolyte for subsequent experiments.

The effect of hydrogen bond catalysis on voltammetric behavior for Xa and HXa at the PAIUCPE

Fig. 2 shows the electrochemical response character-

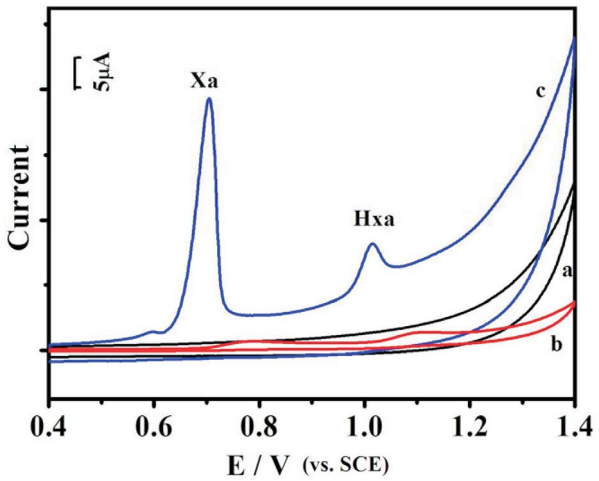


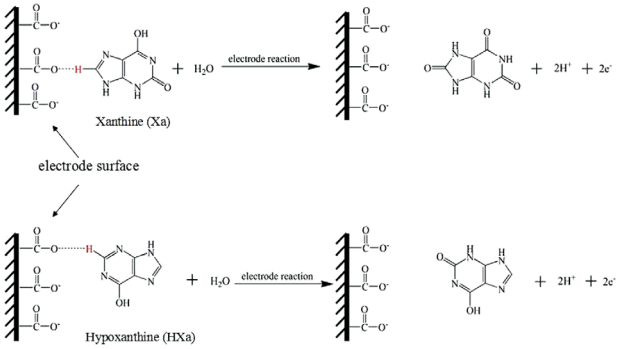
Fig. 2. Cyclic voltammograms of Xa (1.0×10^{-5} mol/L) and HXa (1.0×10^{-5} mol/L) on different electrodes. a-PAIUCPE no containing Xa and HXa; b-IUCPE; c-PAIUCPE; scan rate: 100 mV/s; supporting electrolyte: PBS (pH 6.45); concentration potention: +0.40 V; concentration time: 50s.

istics of a mixture of 1.0×10^{-5} mol/L of Xa and HXa on different electrodes in 0.10 mol/L KH_2PO_4-Na_2HPO_4 buffer solution (pH 6.45). No reduction peaks appear at the IUCPE and PAIUCPE, which means that electrode processes of Xa and HXa are irreversible. Also, as could be seen from Fig. 2, two weak and broad oxidation peaks corresponding with the oxidation of Xa and HXa respectively appear at 0.791 V and 1.118 V at the IUCPE, and their peak potential separation is about 427 mV. At the PAIUCPE, Xa and HXa respectively reveal a well-defined and sensitive oxidation peak at 0.705 V and 1.016 V, and their oxidation peak potentials separately shift negatively by 86 mV and 102 mV, corresponding peak currents increase by 31 and 6.5 times, respectively. This result is also consistent with the principle that the lower the oxidation peak potential was, the lower the activation energy of the reactions, the easier to be oxidized. The reason is due to that the negatively charged oxygen-containing functional groups (carboxyl (–COOH), phenolic hydroxyl (-OH)) at the PAIUCPE surface can interact with electroactive groups of Xa and HXa by hydrogen bonding. Also, it is the hydrogen bond catalysis[23] that reduces the activation energy of electrode reaction for Xa and HXa, accelerates the electron transfer rates of Xa and HXa, promotes the electrochemical oxidation of the Xa and HXa at the surface of PAIUCPE. Moreover, in contrast with the IUCPE, the PAIUCPE surface has more active groups that could form hydrogen bonds with the Xa and HXa, as a result, the peak potentials for oxidation of Xa and HXa shift negatively and their peak currents increase. This implies that the hydrogen bond plays an important role in depressing the energy of reaction.

It could be deduced from the above discussions that the stronger the ability of formation hydrogen bonding, the greater the oxidation peak potential should shift negatively. Compared with the molecule structure of Xa (seen Scheme 1), a slight positive charge of hydrogen atom in 2-position of HXa should be greater than that of hydrogen atom in 8-position of Xa. Therefore, the ability that the hydrogen atom in 2-position of HXa forms hydrogen bond with the negatively charged active groups at the PAIUCPE surface should also be greater, leading to that the oxidation peak potential shifted negatively degree for HXa is greater.

In order to discuss interaction between the tagets (Xa, HXa) and PAIUCPE surface by hydrogen bonding, With the carboxyl anions (-COO^-) at the surface of PAIUCPE, for example, the interaction mechanism can be shown as Scheme 1:

Scheme 1 Electrode reaction mechanisms of hydrogen bond catalysis



According to Scheme 1

I. Assuming that the Xa and HXa are adsorbed at the surface of PAIUCPE through electrostatic (or hydrogen bond) interaction, and the adsorption reactions could be expressed as follows:

$$Xa_{(s)} \rightleftharpoons Xa_{(ads)}$$
$$[Xa]_{(s)} \cdot K_{ads,Xa} = [Xa]_{(ads)}$$

Similarly,

$$HXa_{(s)} \rightleftharpoons HXa_{(ads)}$$
$$[HXa]_{(s)} \cdot K_{ads,HXa} = [HXa]_{(ads)}$$

"$[Xa]_{(s)}$ and $[HXa]_{(s)}$", "$[Xa]_{(ads)}$ and $[HXa]_{(ads)}$" represent respectively the concentration of Xa and HXa in the solution and at the PAIUCPE surface, $K_{ads,Xa}$ and $K_{ads,Hxa}$ represent corresponding adsorption equilibrium constants.

II. The electrode reaction of Xa and HXa adsorbed at the PAIUCPE surface could be expressed as follows:

$$Xa_{(ads,Red)} \rightleftharpoons Xa_{(ads,Ox)} + 2e^- + 2H^+$$
$$HXa_{(ads,Red)} \rightleftharpoons HXa_{(ads,Ox)} + 2e^- + 2H^+$$

Thus, the following formula could be obtained by the Nernst equation:

$$E_{Xa} = E_{Xa}^\circ + (RT/2F)log([Xa]_{(ads,Ox)}[H^+]^2 / [Xa]_{(ads,Red)} = E_{Xa}^\circ + (RT/2F)log([Xa]_{(ads,Ox)}[H^+]^2 / K_{ads,Xa}[Xa]_{(s,Red)}) \quad (1)$$

$$E_{HXa} = E_{HXa}^\circ + (RT/2F)log([HXa]_{(ads,Ox)}[H^+]^2 / [HXa]_{(ads,Red)} = E_{HXa}^\circ + (RT/2F)log([HXa]_{(ads,Ox)}[H^+]^2 / K_{ads,HXa}[HXa]_{(s,Red)} \quad (2)$$

III. Because the oxidation peak potentials of the Xa and Hxa are linear to pH values which respectively obey the linear equations: $E_{pa}(V) = 1.382 - 0.060\,pH$ and $E_{pa}(V) = 1.058 - 0.061pH$ in the section below, (1) and (2) could also be expressed as follows, respectively:

$$E_{Xa} = E_{Xa}^\circ + 0.060\,log\,(1 / K_{ads,Xa}) + 0.060\,log\,([Xa]_{(ads,Ox)} / [Xa]_{(s,Red)}) \mp \{\, 0.060\,pH = 1.382 \mp \{\, E_{Xa}^\circ \mp \{\, 0.060\,log\,K_{ads,Xa} \mp \{\, 0.060\,pH \quad (3)$$

$$E_{HXa} = E_{HXa}^\circ + 0.061log\,(1/K_{ads,HXa}) + 0.061log\,([HXa]_{(ads,Ox)}/[HXa]_{(s,Red)}) \mp \{\, 0.061\,pH = 1.058 \mp \{\, E_{HXa}^\circ \mp \{\, 0.061log\,K_{ads,HXa} \mp \{\, 0.061\,pH \quad (4)$$

Based on the above discussions, the ability of interaction between the hydrogen atom in 2-position of HXa and PAIUCPE is greater than the ability of interaction between hydrogen atom in 8-position of Xa and PAIUCPE. Thus, the $K_{ads,Hxa}$ is greater than $K_{ads,Xa}$, and then the oxidation peak potential shifted negatively degree of HXa is greater than that of Xa.

Could be seen from Fig. 2, the peak currents of Xa and HXa at the PAIUCPE are far more than that of Xa and HXa at the IUCPE, indicating that the stronger hydrogen bonding could improve their peak currents. However, although the $K_{ads,HXa}$ is greater than $K_{ads,Xa}$, the peak current of HXa is much smaller than that of Xa in the same concentration conditions. The reason is that part of Xa not only can be ionized into monovalent anions like HXa, but also 2,6-Dihydroxypurine (Xa) as Xanthine (Xa) tautomers could be ionized into bivalent anions, which makes Xa more easily than HXa migrate towards anode under the action of electric filed in the condition of the same concentration time. In addition, the product of Xa to be oxidized is uric acid with a stable molecular structure of 2,6,8-3-ketone groups, and the product of HXa to be oxidized is Xa with less stable molecular structure of 2,6-2-ketone groups. Consequently, the sizes of peak currents for oxidation of Xa and HXa are not only related to the intensity of hydrogen bond formation, but also related to the properties of product.

The pH effects on the peak potential and peak current for oxidation of Xa and HXa

As shown in Fig. 3, the oxidation peak potentials (E_{pa}) of HXa and Xa shift negatively with the increase in pH. Also, there is a linear relationship between oxidation peak potential and pH values, and the linear regression equations are: $E_{pa}(V) = 1.058 - 0.061pH$ (R = -0.9999) and $E_{pa}(V) = 1.382 - 0.061pH$ (R = -0.9994), respectively. The slopes of both linear equations are very close to the theoretical value of -59 mV/pH, suggesting that the electrode reactions involve the same numbers of proton and electron. Meanwhile, the peak currents for the oxidation of HXa and Xa also decrease with the increment of pH in the range of

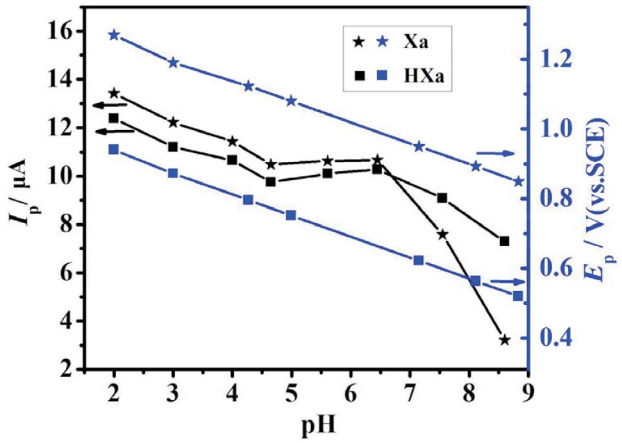


Fig. 3. The effect of pH on the peak current and peak current of oxidation for Xa and HXa. Supporting electrolyte: PBS (pH 6.45); scan rate: 100 mV/s.

pH = 2.00-4.65. The reason may be that nitrogen atoms in the HXa and Xa molecules could be intensely attracted by a positively-charged (H^+) "Hemholtz" layer as mentioned above at higher acidity conditions. Also, the total amounts of HXa and Xa diffusing to the reaction layer increase with the enhancement of the solution acidity. Consequently, their peak currents are the largest at the pH = 2.0. With the increase in pH, the deprotonation in the "Hemholtz" layer weakens the abilities of HXa and Xa to be adsorbed by PAIUCPE surface, which lowers the concentration of HXa and Xa in the reaction layer, so that their peak currents decrease.

However, the peak currents increase slightly again with pH increasing in the range of pH = 4.65-6.45, and achieve their greater values at pH = 6.45. This might be due to that the solutions of pH = 4.65-6.45 have all become slightly acidic, corresponding to the degree of protonation in the "Hemholtz" layer and the amounts of the HXa and Xa diffused to reaction layer should have little difference under these conditions, which make the peak currents remain basically unchanged. The reason that the peak currents increase slightly with increase in pH might be related to that oxidation potentials of HXa and Xa shift negatively. Usually, owing to the lower oxidation potential of reducing agent, the easier it is oxidized.

When pH value exceeds 6.45, their peak currents obviously decrease again as the raise of pH value from 6.45 to 8.60. Accordingly, the solution has turned from the nearly neutral into weak alkaline, the degree of protonation in the "Hemholtz" layer should also has little difference in the range of pH = 6.45-8.60. Their peak currents should not once more obviously be decreased with the increase in pH. As a matter of fact, once pH value exceeds 6.45, peak currents for oxidation of HXa and Xa unexpectedly begin to drop with the rise in pH value, which is inconsistent with the principia of which the lower the oxidation potential of reducing agent, the easier it to be oxidized theoretically. The reason may be because the sizes of peak currents for oxidation of HXa and Xa are also affected by the distribution coefficients of their different species and the characters of PAIUCPE.

In the light of the pKa (5.4) of hydroxyl of UA,[24] assuming that the dissociation constants (pKa) of hydroxyl of the Xa and HXa are the same as pKa (5.4) of UA on the basis of their similarity molecular structure, the distribution coefficients for the monovalent anions of Xa and HXa all equal to 92% and are near to 100% at pH = 6.45 and pH ≥ 7.4, respectively. Also, assuming that the dissociation constant (pKa) of the carboxyl (-COOH) at the PAIUCPE surface is the same as the pKa (4.74) of acetic acid. According to the calculation of acetic acid distribution coefficient, it could be known that the carboxyl (-COOH) could be completely ionized to its anion ($-COO^-$) on the PAIUCPE surface at pH ≥ 6.74. Therefore, the monovalent anion of both Xa and HXa would be subjected to the repulsive interaction of negative charge at the PAIUCPE surface, while the repulsive interaction would impede the oxidation reactions of Xa and HXa at the surface of PAIUCPE, this leads to the decrease of the peak current as the pH increasing. In addition, in the alkaline solution, such the active groups as the phenolic hydroxyl group at the PAIUCPE surface would also be ionized into corresponding anion, which would further weaken the interactions of hydrogen bonding between the PAIUCPE surface and the analytes (Xa, HXa), and then the amounts of Xa and HXa in the reaction layer would not only reduce, but also the hydrogen bond catalysis also would decrease. As a result, their peak currents markedly drop with the increment of pH at pH > 6.45.

The effect of scan rate on the peak current for oxidation of Xa and HXa

The oxidation peak current (I_{pa}) increases gradually with the scan rate increasing varied from 10 to 450 mV/s. In the range of 10-100 mV/s, the oxidation peak currents of Xa and HXa are linearly to the square root of scan rate ($v^{1/2}$) with the linear equations of $I_{pa}(\mu A) = -2.04 + 34.27v^{1/2}$ (V/s) (R = 0.9924) and $I_{pa}(\mu A) = -1.71 + 27.64v^{1/2}$ (V/s) (R = 0.9930), which accord with the Randles-Sevcik equation. As could be seen from Fig. 4, when the scan rate is greater than 100 mV/s, the oxidation peak currents of Xa and HXa increase with increase of $v^{1/2}$ deviated from the straight toward the trend of upwarp. The results suggest that their electrochemical oxidation processes are typically controlled by diffusion at lower scan rate while adsorption characteristic at higher scan rate.[25] The better signal-to-noise ratios for the determination of Xa and HXa could be obtained at a scan rate of 100 mVs^{-1}, therefore, it is chosen for the subsequent experiment.

The effect of concentration time and concentration potential on the peak current for oxidation of Xa and HXa

The influence of different concentration potential on the sizes of oxidation peak currents of Xa and HXa is investigated under stirring in the range of +0.10 to +0.40 V. Results show that the change of concentration potential has

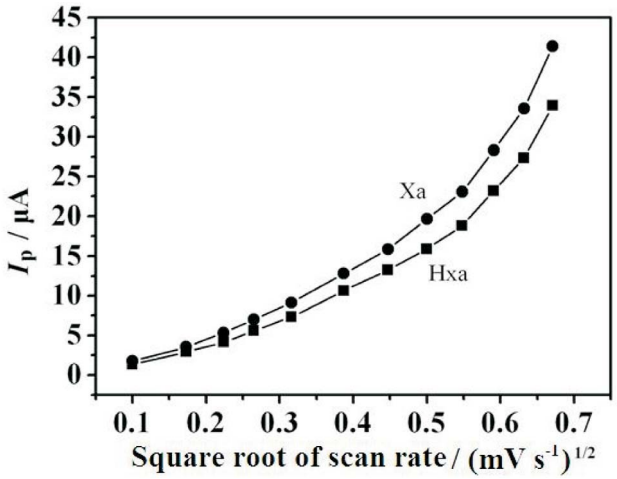


Fig. 4. The relationships between oxidation currents of Xa (1.0×10^{-5} mol/L) and Hxa (1.0×10^{-5} mol/L) vs the square roots of scan rate. Supporting electrolyte: PBS (pH 6.45).

little influence on the size of oxidation peak current. The concentration potential of +0.4 V is chosen for the subsequent experiment.

As shown in Fig. 5, keeping the concentration potential of +0.4 V constant, when the concentration time increases from 0 to 90s, the oxidation peak current of Xa reaches the maximum at t = 50s, then decreases slowly with the continuing increase of concentration time. The oxidation peak current of HXa also declines slowly when concentration time is greater than 10s. Optimal concentration time of 50s is employed for the subsequent experiments.

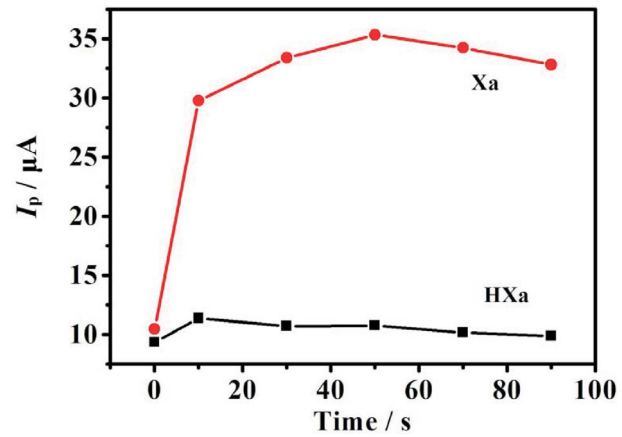


Fig. 5. The effect of concentration time on the oxidation current of Xa and HXa. C(Xa) = 1.0×10^{-5} mol/L; C(HXa) = 1.0×10^{-5} mol/L; supporting electrolyte: PBS (pH 6.45).

Stability and reproducibility of PAIUCPE

In order to characterize the reproducibility of the PAIUCPE, a series of seven repetitive measurements are carried out for 1.0×10^{-5} mol/L mixture of Xa and HXa with the relative standard deviations (R.S.D.) of 1.5% and 2.9%, respectively. After the PAIUCPE is stored at 4 °C for a week, the oxidation peak currents of Xa and HXa experience only a small decrease and the sensitivity remains more than 97% of the initial signals with the relative standard deviations (R.S.D.) of 2.1% and 1.7%, respectively. All the results indicate that the PAIUCPE has excellent reproducibility and stability.

Simultaneous determination of Xa and HXa

In order to further verify the feasibility for simultaneous determination of the Xa and HXa at the PAIUCPE, quantitative analysis of the Xa and HXa in the mixed solution is carried out by LSV when the concentration of Xa and HXa changes simultaneously. It could be seen from Fig. 6 that two well-defined oxidation peaks appear at about +0.705 and +1.016 V corresponding to the oxidation of Xa and HXa, respectively. The oxidation peak current (I_{pa}) increases linearly with the concentration of Xa in a range of 6×10^{-8}-1.5×10^{-5} mol/L, and the linear regression equation is I_{pa}(μA) = 0.0680 + 3.21c (μmol/L) (R = 0.9954). The linear regression equation of HXa is I_{pa}(μA) = 1.85 + 0.592c (μmol/L) in the range of 2×10^{-7}-5×10^{-5} mol/L with the linearly correlation coefficients of 0.9925.

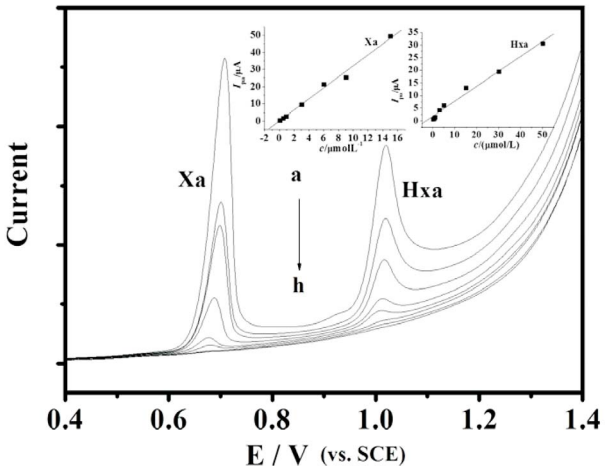


Fig. 6. Linear sweep voltammetry for the simultaneous determination of Xa and HXa, C(Xa) (a–h): 0.06, 0.1, 0.5, 0.9, 3, 6, 9, 15 μmol/L; C(HXa) (a–h): 0.2, 0.5, 0.9, 3, 5, 15, 30, 50 μmol/L. Insert: linear relationship of I_{pa} vs concentration of HXa and Xa; supporting electrolyte:PBS (pH 6.45).

In order to evaluate the applicability of the proposed method for analysis of Xa and HXa, the PAIUCPE is further employed for determination of human urine samples. Because the electrochemical signal of Xa is very little and the content of HXa in human urine is lower than the detection limit of this method, the real samples are spiked with certain amount of Xa and HXa. Two urine samples are quantitative determination by standard-addition method. The results are summarized in Table 1.

Table 1. The determination of Xa and Hxa in human urine sample and the recovery (n = 5)

Sample	Original (μmol/L)		Added (μmol/L)		Found (μmol/L)		R.S.D %	
	Xa	Hxa	Xa	Hxa	Xa	Hxa	Xa	Hxa
1	2.20	4.72	2.00	2.00	4.16	6.76	3.44	2.81
			4.00	4.00	6.30	8.58	3.36	1.84
2	1.95	5.51	2.00	2.00	4.00	7.52	1.78	2.50
			4.00	4.00	6.03	9.38	2.54	1.90

EXPERIMENTAL

Apparatus and reagents: Cyclic voltammetry (CV) and linear sweep voltammetry (LSV) were performed by CHI660C electrochemical workstation (Shanghai Chenhua Instrument Company, China). A PFS-80 digital pH meter (Shanghai dazhong analysis instrument Company, Shanghai, China) was used for preparation of the buffer solutions. A three-electrode system was comprised of PAIUCPE working electrode, platinum counter-electrode and a saturated calomel reference electrode (SCE). All the potentials in the text were quoted versus this reference electrode.

Graphite powder (The purity is 99.85%, purchased from Shanghai, China) and Liquid paraffin (C.P purchased from Xinxiang, China) were used for the preparation of PAIUCPE. Xanthine (Sigma) and hypoxanthine (Sinopharm Chemical Reagent Co. Ltd.) stock solution of 1.0×10^{-3} mol/L were prepared as required in 0.10 mol/L NaOH and preserved at 4 °C in the dark, respectively. Na_2HPO_4 and KH_2PO_4 were used for the preparation of 0.10 mol/L buffer solutions. All other chemicals were at least analytical-reagent grade and all solutions were prepared with doubly distilled de-ionized water. All experiments were carried out at room temperature.

Fabrication of the pre-anodized inlaying ultrathin carbon paste electrode (PAIUCPE): An industry leftover 316L stainless steel rod with 2.5 mm diameter and a known length was sealed in a plastic tube of matching length. One end of the rod was used as the electrode connection held out of the plastic tube and the other one as the working electrode surface. Prior to use, the surface of the working electrode was polished with 0.05 mm alumina slurry for 8–10 min, washed with 1:1 (V/V) nitric acid, absolute ethanol and double distilled water in an ultrasonic bath for 5 min, respectively, and allowed to dry in air. A 7:3 (w/w) mixture of graphite powder and paraffin was blended in an agate mortar and ground for 20 min until a homogeneous paste was obtained. Then the pretreated 316L substrate was rubbed in the carbon paste to fabricate an inlaying ultrathin (about thickness of 100 nm) carbon paste electrode (IUCPE).[21] The as-prepared IUCPE was anodized by successive scan for 30 cycles from 0 V to +1.2 V with a scan rate of 100 $mV \cdot s^{-1}$ in 0.20 $mol \cdot L^{-1}$ of NaOH solution. After pre-anodization, it was thoroughly rinsed with double distilled deionized water and dried in air, and then the PAIUCPE was fabricated for subsequent tests.

Experimental procedure: A certain volume of Xa and HXa standard solution was transferred into a 10 mL colorimetric tube and diluted to degree scale with buffer solution of PBS (pH = 6.45), and the three-electrode system was installed in the cell. The voltammetric behaviors of Xa and HXa were studied in 0.10 M PBS (pH = 6.45) at the PAIUCPE between +0.4 to +1.4 V at a scan rate of 100mV/s by linear sweep voltammetry. All experiments were accomplished at room temperature.

CONCLUSIONS

In this paper, the PAIUCPE was prepared by simple and fast electrochemical pretreatment. Moreover, the mechanisms of interaction between the analytes (Xa, HXa), probe reagent $Fe(CN)_6^{4-}$, supporting electrolytes and the active groups at the PAIUCPE surface are investigated via a detailed control experiments and disccusion. Especially, it is proposed that the hydrogen bond plays an important role in depressing the activation energy of electrode reaction. In addition, this proposed method could be successfully used for simultaneous determination of Xa and HXa in human urine samples with the satisfactory results.

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