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Imprinted Poly(*o*-phenylenediamine-co-aniline) Electrode for Warfarin Assay in Human Samples by Differential Pulse Voltammetry

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In the present paper, a modified electrode utilizing molecularly imprinted polymer (MIP) of *o*-phenylenediamine-co-aniline (*o*-PDA-co-AN) was employed for the measurement of warfarin. The effective factors on the electrode response such as ratio of aniline to *o*-phenylenediamine concentration, warfarin content as a template, thickness of the polymer, pH of measurement solution, accumulation time and potential applied to electrode, were optimized. Measurement was performed in three stages; electrode preparation, warfarin accumulation and differential pulse voltammetry within the potential range of 0.0 to -1700 mV. Present findings showed that the peak pertaining to the reduction of C=O double bond was observed at about -1280 mV, in Britton-Robinson buffer with pH = 2.0. The calibration plot was linear over the concentration range of 10^{-6}-10^{-4} M of warfarin sodium in Britton-Robinson buffer at pH = 2.0 with sensitivity of 89.8 nA μM^{-1} and good repeatability lower than 7.7 %. Limits of detection and quantitation for seven sequential measurements of blank solution were 6.22×10^{-8} and 2.05×10^{-7} M, respectively. The modified electrode was successfully used for warfarin measurement in pharmaceutical formulation and human serum.

Key Words: Warfarin, Voltammetry, Imprinted Polymer, Modified Electrode.

INTRODUCTION

Warfarin sodium, also called [RS-4-hydroxy-3-(3-oxo-1-phenylbutyl) coumarin sodium][1,2], is a widely used oral anticoagulant prescribed for prevention of blood clot formation and treatment of arterial or venous thromboses or emboli[3]. Coumarin is a toxic, sweet chemical compound found in many plants, which are notorious for causing bleeding and abortions in cattle and other animals. It has been used as antirodents[4]. Warfarin is an antagonist of vitamin K, hence preventing synthesis of coagulation factors II, VII, IX, X and protein C from the liver[5].

Measurement of warfarin sodium as an anticoagulant drug in the pharmaceutical and biological samples is of supreme importance because of its narrow therapeutic index[6]. To do this, there have been a number of methods including square-wave adsorptive cathodic stripping voltammetry[1], potentiometry and potentiotitrimetry[2],

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HPLC[3,7], capillary electrophoresis[8], supercritical fluid chromatography/mass spectroscopy[9], HPLC-electrospray ionization mass spectroscopy[10], liquid chromatography[11], spectrophotometry[12] and phosphorescence[13]. Although these technologies have high sensitivity, they suffer from unwieldy instruments and tedious procedures. Except the method using hanging mercury drop electrode[1], there have not been great numbers of electrochemical studies about warfarin measurement.

Electropolymerization of the various functionalized monomers can lead to the modified electrodes having the properties of the analyte accumulation[14-17]. Copolymerization is a simple way to preparation of new polymers and greatly increases the ability of polymer scientists to tailor make a material with specifically desired properties[18,19].

Molecular imprinted polymers (MIPs) have high affinity for the template and are widely used for making sensors and for analytical as well as catalytic purposes[20]. So, the aim of this study is to determine the amount of warfarin in drug formulations (tablets) and human serum using an electrode modified by a copolymer imprinted with warfarin molecules as template.

EXPERIMENTAL

In present work, all of the tests were performed using a Metrohm electrochemical analyzer model 746 VA Trace Analyzer. The reference electrode was Ag/AgCl, the working and counter electrodes were glassy carbon (Azar Electrode, Iran) hanging mercury drop electrode, Bi film and platinum rod, respectively. Deoxygenation of the solutions was performed by pure nitrogen gas for 180 s. The pH measurement was carried out by PTR 79 pH meter (ZAG Chemic, Iran) and EDT glass electrode. The glassy carbon electrode was polished with 0.3 μm alumina powder and rinsed with distilled water to yield a smooth shiny surface before performing tests. All of the chemicals used (all from the Merck, except warfarin sodium, which was obtained from Sigma) were of analytical purity and for solutions preparation, double-distilled water was used throughout. Necessary solutions (Britton-Robinson, 0.04 M, 0.2 M NaOH, *o*-PDA/AN polymerization solution, warfarin sodium from 1.0×10^{-7}-1.0×10^{-4} M and benzyl trimethyl ammonium chloride) were prepared according to ordinary standard methods.

Electrode modification: Glassy carbon electrode was polished with alumina powder and then covered by poly (*o*-PDA-co-AN) *via* cyclic voltammetry in 4.0 mM co-monomers and 0.5 M sulfuric acid and 0.03 M warfarin as template molecule. Deoxygenation was achieved by pure nitrogen gas for 180 s. In 40 cycles of cyclic voltammetry from +1.4 to -0.1 V with a scanning speed of 100 mV/s, the polymer is formed on glassy carbon electrode. The electrode was rinsed in distilled water and placed in 1.0 M benzyl trimethyl ammonium chloride for 1 h, to extract warfarin from polymer. The electrode was placed in Britton-Robinson buffer solution with pH = 2.0 and applied 5 cycles of cyclic voltammetry from +1.0 to -1.0 V, in order to remove the remaining template and stabilize the polymeric covering.

Electrochemical measurements: The electrogenerated molecularly imprinted poly (*o*-PDA/AN) electrode was placed in a B-R 0.04 M buffer solution at pH = 2.0 and the differential pulse voltammograms of electrode in the potential range of 0.0 to -1700 mV at the scan rate of 100 mV/s, before and after accumulation of warfarin, were obtained. High purity nitrogen gas was purged into the electrochemical cell for 180 s before each measurement and during the measurements.

Determinationt of warfarin in tablets and human serums: Five hemofarin tablets of 5 mg warfarin sodium was dissolved in distilled water, shaken for 5 min, filtered and a solution prepared with a concentration of 1.6×10^{-3} M. After diluting it to 5.0×10^{-6} M and buffering to pH = 2.0 with Britton-Robinson buffer, the modified electrode was placed in it for 30 s with application of -700 mV potential. Then the electrode rinsed with distilled water and transferred in the Britton-Robinson buffer solution for obtaining the voltammogram of warfarin entrapped in the polymer matrix.

In order to measuring the warfarin spiked into the human serum, collected serum samples from volunteers were mixed and stored at -7 ºC until analysis. The analyte sample was prepared by adding 0.5 mL serum sample, 0.5 mL 5×10^{-5} M warfarin sodium and 4.0 mL ethanol (to precipitate the proteins). 5 aliquots of the sample were centrifuged at 6000 rpm for 10 min and precipitated proteins separated. Each sample was diluted to 10 mL by Britton-Robinson buffer and tested *via* the optimized procedure.

RESULTS AND DISCUSSION

At first, a hanging mercury drop electrode (HMDE, as working electrode, area: 0.031 cm^2) and Ag/AgCl saturated with potassium chloride as reference, platinum rod as opposite electrode and a warfarin solution (5.0×10^{-6} M) were used to get the differential pulse voltammogram in the range of 0.0 to -1600 mV as shown in Fig. 1. The observed peak in -1448 mV is related to reduction of carbonyl functional group in warfarin and shifts to more negative value with increasing the pH of buffer solution. This is in exact agreement with the results obtained by Ghoniem *et al.*[1].

Recently bismuth and bismuth film electrodes have found a wide range of applications in anodic stripping voltammetry[21] cathodic adsorptive stripping voltammetry of metal ions[22] and a few studies for determination of organic compounds[23]. Bismuth electrodes exhibit favourable electrochemical properties comparable to commonly used mercury electrodes and thus represent attractive and low toxic alternative in replacement of mercury. For this reason, bismuth-film electrodes were prepared by the electrical formation of bismuth on gold electrode in an acetate buffer[23] and also in HCl solution consisting of bismuth nitrate[23] and then used as working electrode for accumulation and voltammetric determination of warfarin according to the conditions used in the case of HMDE. Response of the electrode in Britton-Robinson buffers at various pH containing warfarin sodium revealed that bismuth film electrode (unlike mercury) has no surface adsorption for warfarin and is not suitable for measurement of warfarin (Fig. 1b).

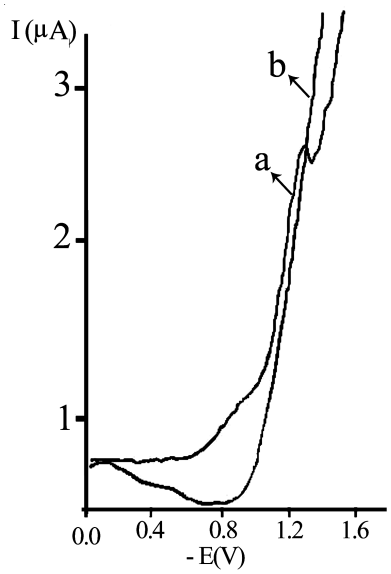

Fig. 1. Differential pulse voltammograms for 5×10^{-6} M warfarin sodium in a B-R buffer (pH = 5.0) with pre-concentration time of 30 s in -0.7 V pre-concentration potential at scan rate of 100 mV/s; at (a) HMDE and (b) Bi-film electrode. Pulse amplitude = 50 mV, pulse width = 40 ms

The development of molecular devices capable of recognizing pesticides and drugs through their size, shape and functional group distribution is becoming one of the most important fields in analytical chemistry[20]. Molecular imprinting offers the possibility of synthesizing a tailor-made polymer for a target analyte for which a natural receptor may not exist or may be very difficult to obtain. Molecular imprinted polymer gains its binding ability by the pre-organized cavities, which were formed by self-assembling polymerizable monomers around a template[20].

Different types of polymers such as polypyrrole, overoxidized polypyrrole, polyindole, over-oxidized polyindole and poly (*o*-PDA/AN) was synthesized (in the presence of warfarin as template molecule) that can be easily prepared from the aqueous and organic solutions of their monomers. Among these polymers, poly(*o*-PDA/AN) showed that is able to incorporate the warfarin template. This last one also has successfully employed in other studies to entrap enzymes[24], determination of glucose[25], sorbitol[26] and metamitron[20].

In order to demonstrate that the electrode is able to accommodate the warfarin molecules inside the vacations, electrode was treated in benzyl trimethyl ammonium chloride for 1 h and preconditioned in B-R 0.04 M buffer pH = 2.0 by 5 cycles of potential sweep between +1.0 and -1.0 V. The voltammogram of the electrode was recorded in B-R buffer solution (Fig. 2a). It can be observed that after removing the warfarin molecules no cathodic current appears related to the reduction of the C=O double bond in analyte molecules.

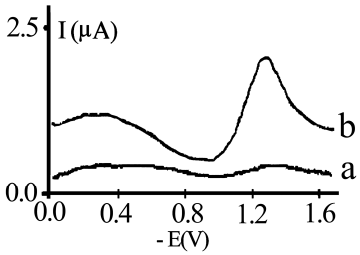

Fig. 2. Differential pulse voltammograms corresponding to the reduction signal of the entrapped template measured by (a) a MIP electrode after extracting the template (b) a MIP electrode after accumulation of 5×10^{-6} M warfarin sodium in a Britton-Robinson buffer (pH = 2.0) for 60 s at -700 mV accumulation potential. Measurements made in a Britton-Robinson 0.04 M buffer solution of pH 2.0 at a scan rate of 100 mV/s. Pulse amplitude = 50 mV, pulse width = 40 ms

After that, the electrode was placed into the warfarin solution for 1 min and then rinsed with distilled water and voltammogram was obtained in Britton-Robinson 0.04 M buffer solution (pH = 2.0) (Fig. 2b). Peak at -1280 mV is due the reduction of C=O bond, appearing at potentials about 170 mV easier than that of HMDE and then having less possibility of interfering effects.

Optimization of the conditions of the procedure was done by changing each affecting parameter separately, with at least 5 repeats for each measurement. Table-1 shows a brief summary of them.

TABLE-1
PARAMETERS AFFECTING CURRENT OF PEAK
VOLTAMMOGRAM AND THE OPTIMAL CONDITION

Parameter	Tested range	Optimal condition
pH of B-R buffer accumulation solution	0.5-6	2.0
Number of polymer cycles	10-50	40
Accumulation time (s)	0-45	30
Accumulation potential (preconcentration) (mV)	-700-+700	-700
Mole ratio of AN/*o*-PDA	0.25-1.25	1:1
Concentration of warfarin in the polymerization solution (M)	0.007-0.04	0.03

As previously reported by other researchers[20] the ratio of AN to *o*-PDA is very important when preparing a MIP based modified electrode. Then, a series of experiments were carried out keeping the concentrations of *o*-PDA and warfarin constant and changing the concentration of AN. Fig. 3 shows that the best results were obtained *via* employing a ratio of 1:1 *o*-PDA to AN monomers. As the value of the ratio is higher, the insulating properties of the prepared polymer inhibits the polymer grows and at lower ratios the polymer will be rich of AN and is highly electroactive with the resulting loss of selectivity of the MIP for the template[20]. For this reason the peak current related to the template incorporated into the polymer matrix decreases.

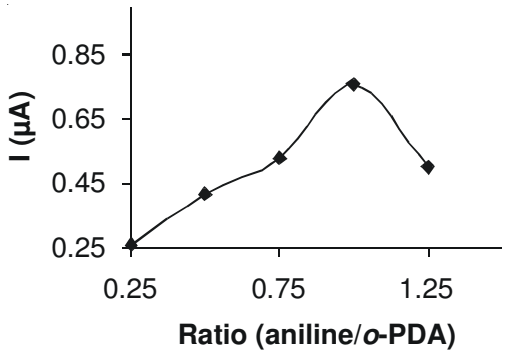

Fig. 3. Reduction signal of the entrapped template in MIP electrode after being synthesized with different *o*-PDA: aniline ratios. The other conditions are similar to those illustrated in Fig. 2

Fig. 4 shows the dependence of electrode response on the pH of measurement solution. It can be seen that the peak current is pH dependent and the best results achieved in Britton-Robinson 0.04 M buffer solution at pH 2.0. At pH lower than 2, warfarin molecules and also polymer are highly protonated and it can repulses the warfarin molecules from surface of the electrode. At higher pHs, electroactivity of the polymer diminishes so that at pH more than 4.0 it is converted to a non-electroactive non-conducting polymer film[18] and losses its ability to transport the electric charge.

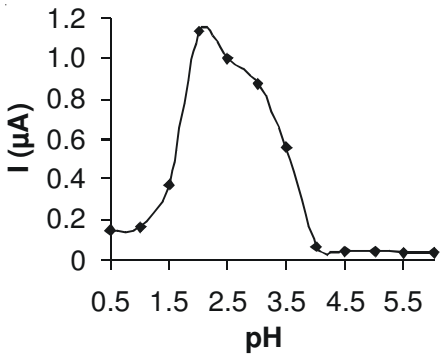

Fig. 4. Effect of pH of measurement solution on the peak current of differential pulse voltammograms for 5×10^{-6} M warfarin sodium. The other conditions are similar to those illustrated in Fig. 2

Fig. 5 shows the effect of number of cyclic scans applied in the electropolymerization step which is directly related to the thickness of the polymer adhered on the surface of the electrode. The more thickness of the polymer (up to 40), the more template molecules incorporate into the matrix and the greater reduction peak current will be observed and after that reaches to a stable value.

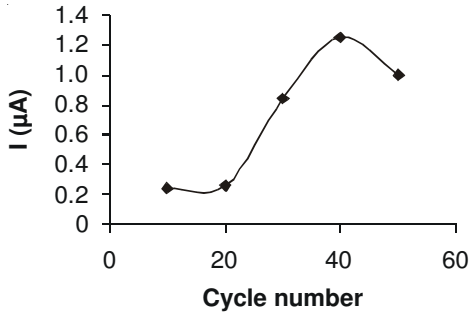

Fig. 5. Influence of the applied number of cyclic scans of polymerization in the reduction signal of entrapped template. All conditions are similar to those illustrated in Fig. 2.

The reduction peak currents increases on the increase of accumulation time period up to 30 s and then decreases (Fig. 6). It shows that the complete accommodation of template molecules is gradually being achieved.

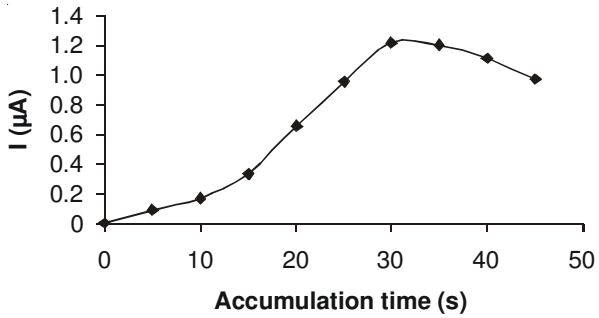

Fig. 6. Influence of the accumulation time period on the peak current of differential pulse voltammogramms for 5×10^{-6} M warfarin sodium. The other conditions are similar to those illustrated in Fig. 2

As the accumulation potential varies from -700 and +500 mV, the peak current reduces rapidly (Fig. 7). Negative potential applied to the MIP modified electrode increases the attraction forces on the protonated warfarin molecules in the acidic medium and therefore, causes the higher accumulation of warfarin molecules.

The amount of template was optimized to be 0.03 M (Fig. 8). When the concentration of the warfarin was increased up to 0.03 M, the incorporation of the warfarin molecules inside the polymer matrix increases, however after that it probably inhibits the polymer formation on the surface of the electrode. Therefore, the thickness of the polymer reduces and consequently the peak current of template becomes smaller. By using the MIP electrode at optimized condition a linear calibration graph was obtained over the concentration range of 1.0×10^{-6}-1.0×10^{-4} M warfarin sodium with a sensitivity of 89.9 nA mM^{-1} (r = 0.993). The LOD and LOQ were found to equal 6.2×10^{-8} and 2.0×10^{-7} M warfarin sodium, respectively.

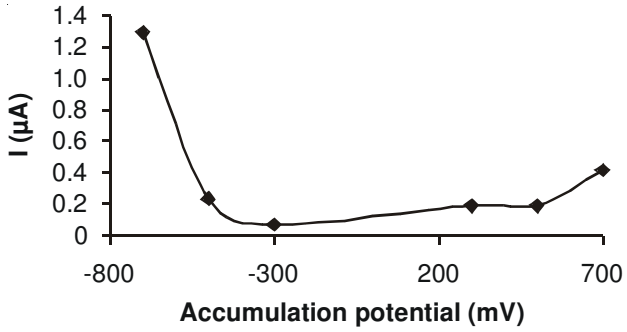

Fig. 7. Effect of accumulation potential on the peak current of differential pulse voltammograms for 5×10^{-6} M warfarin sodium. The other conditions are similar to those illustrated in Fig. 2

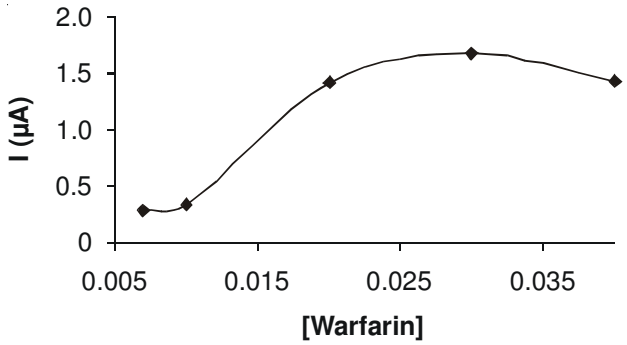

Fig. 8. Effect of concentration of template in the polymerization solution on the peak current of differential pulse voltammograms 5×10^{-6} M warfarin sodium. The other conditions are similar to those illustrated in Fig. 2

The proposed method was successfully employed for analysis of hemofarin tablets and warfarin sodium spiked in human serum. The mean response of five replicate measurements on samples prepared from warfarin tablets was a peak current of 1.7 µA. The calibration line equation showed that warfarin concentration was 4.8×10^{-6} which is corresponding to 96.0 ± 2.2 the mean percentage recovery. The mean percentage recovery for five replicate measurements of human serum samples spiked with warfarin sodium was found to equal 89.2 ± 2.3.

Conclusion

Present findings showed that the peak pertaining to the reduction of C=O double bond was observed at -1280 mV by the polymer modified electrode, in comparison with -1450 mV which was observed at the surface of HMDE. It means that the reduction peak has shifted to the 170 mV lower potential which reduces the risk of intervention of interfering species. The calibration plot was linear over the concentration range of 1.0×10^{-6}-1.0×10^{-4} M warfarin in B-R buffer with pH = 2.0. Limits

of detection and quantitation for seven sequential measurements in a blank solution were obtained to be 6.2×10^{-8} and 2.0×10^{-7} M, respectively. The modified electrode used for warfarin measurement was successfully applied on pharmaceutical formulation and human serum.

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