Research Article

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Determination of Acetaminophen Using a Glassy Carbon Electrode Modified by Horseradish Peroxidase Trapped in MWCNTs/Silica Sol-Gel Matrix

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A B S T R A C T

A new and easy way to fabricate voltammetric biosensor for acetaminophen determination was developed based on horseradish peroxidase (HRP) trapped between silica sol-gel film and multi-walled carbon nanotubes on glassy carbon electrode. Acetaminophen determination was carried out in presence of H_2O_2 as enzyme activator. The modified electrode showed excellent electrocatalytic activity and rapid response to acetaminophen in the presence of H_2O_2 as enzyme activator. Various parameters influencing the biosensor performance such as amount of enzyme, H_2O_2 concentration, potential scan rate and pH have been investigated. Under the optimum conditions, a wide linear range of 1.85×10^{-6} to 2.7×10^{-3} M was obtained for acetaminophen determination. Limit of detection was calculated about 18 nM and sensitivity was about 220 nA/µM. Furthermore, the proposed biosensor was successfully examined for simultaneous determination of acetaminophen, uric acid (UA) and folic acid (FA) as prevalent interferes. The proposed biosensor showed satisfied stability for 3 weeks and applicability of developed biosensor was confirmed with accurately evaluation of acetaminophen in real samples such as urine and tablet.

Keywords: Biosensor, Horseradish peroxidase, Acetaminophen, Enzyme immobilization, Multi-walled carbon nanotube, Simultaneous determination.

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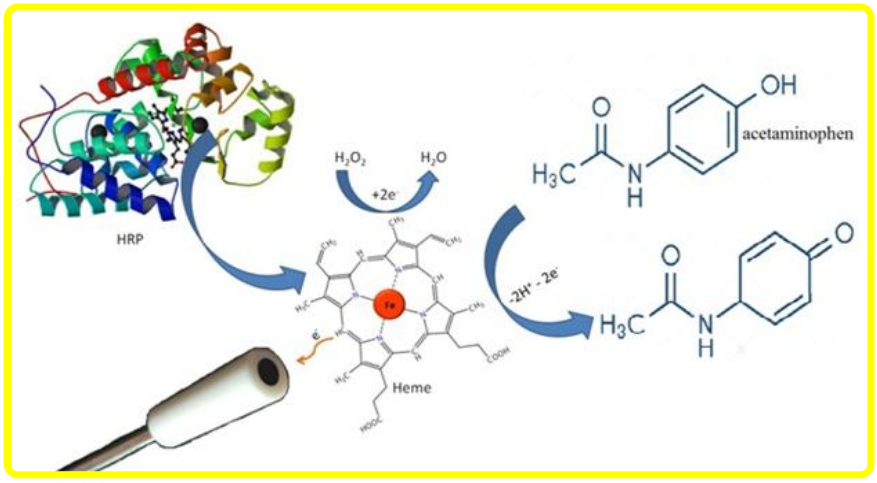


1. Introduction

Acetaminophen, N-acetyl-p-aminophenol, or paracetamol is a common phenolic compound that is commonly used in pharmaceutical formulations to relieve pains and fever caused by a variety of viral or bacterial infection [1]. Normal concentration range of acetaminophen for therapeutic purposes can be affected by several factors such as sex, age, general health, and synergism with other compounds. Nevertheless, its overdose may lead to the accumulation of toxic metabolites, causing severe or fatal hepatotoxicity and nephrotoxicity related to renal failure. Therefore, controlling acetaminophen doses in pharmaceutical formulations is vital in the fields of clinical chemistry and quality control [2]. So far, various techniques such as spectrophotometry [3], liquid chromatography [4], near infrared transmittance spectroscopy [5], voltammetry [6], UV–Vis spectrophotometry [7], Fourier transform infrared spectrophotometry [8] or electrochemical detection techniques [9] have been developed for quantitative measurement of acetaminophen.. Among these techniques, electrochemical detection is the most popular method because it is simple, fast, cost effective and reproducible.

In the field of electrochemical measurement techniques, various electrodes including graphene-modified carbon-paste electrode [10], carbon-coated nickel magnetic nanoparticles modified electrodes [11], boron-doped diamond [12], gold electrodes modified with self assembled monolayer [13], carbon film resistor electrode[14], modified electrode surface with zirconium alcoxide porous gels [15], gold nanoparticle modified carbon paste [16], carbon ionic liquid [17] and MWCNTs/carbon nanoparticle modified electrode [18] have been used in electrochemical determination of acetaminophen. However, to the best of our knowledge, there is no report on the use of horseradish peroxidase (HRP) with multi-walled carbon nanotubes (MWCNTs) and silica sol-gel (for immobilization of HRP) in acetaminophen determination [19]. Carbon nanotubes (CNTs), which is used in current developed electrode, are one of the most common and effective modifiers which has been used to modify carbon paste electrodes. CNTs are gaining popularity in electrochemistry as a viable nanomaterial due to their extraordinary electronic properties, large surface area, significant mechanical strength, mass transfer capabilities, high catalytic capability and chemical and structural characteristics [20, 21].

A selsctive and sensitive electrochemical method for determination of any phenolic compound like acetaminophen is the

application of proxidase enzymes such as laccase [22, 23], tyrosinase [24, 25] and HRP [26, 27] in electrode modification. The current study reports the sensitive determination of acetaminophen with electrocatalytic reduction of acetaminophen by HRP in presence of H_2O_2. Furthermore acetaminophen was simultaneously determined with UA and FA by applying of electrocatalytic oxidation of acetaminophen by HRP in presence of H_2O_2.

2. Experimental

2.1. Materials

Horseradish Peroxidase (EC.1.11.1.7, 250 Umg^{-1}) was purchased from Sigma-Aldrich and was used as received. Tetra ethoxy silane (TEOS) was obtained from Merck. MWCNTs with the average diameter of 20–60 nm were purchased from Neutrino Co. (Iran). Raw material of Acetaminophen was from Darou Pakhsh Co. (Iran). All other reagents were of analytical grade. Phosphate buffer (PB) (50 mM) were prepared from H_3PO_4, NaH_2PO_4 and Na_2HPO_4 and pH values were adjusted by HCl and NaOH solutions. The solutions were prepared with deionized water and deoxygenated by bubbling high purity (99.99%) nitrogen gas through them for 15 min prior to the experiments. All experiments were carried out at room temperature.

2.2. Apparatus

Voltammetric measurements were performed using a three electrode set-up, including the modified glassy carbon electrode (GCE), GCE/MWCNTs/HRP/silica sol-gel, as working electrode, an Ag/AgCl (3.0 M KCl) as reference electrode, and a platinum foil as counter electrode. Voltammetric measurements were performed using a computer-controlled μ-Autolab modular electrochemical system (PGSTAT101, the Netherlands), driven with NOVA Software (upgrade 1.10).

2.3. Fabrication of GCE/MWCNTs/HRP/silica sol-gel

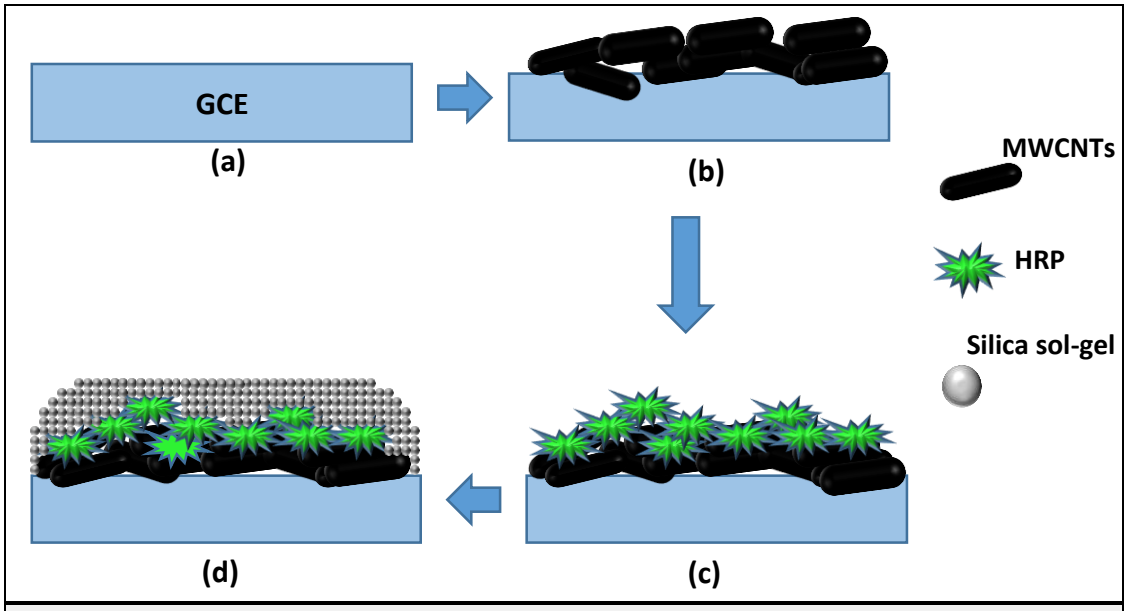
10 mg of native HRP was dissolved in 1.0 mL of 50 mM PB. 15 mg of MWNTs was dispersed in 10 mL of acetone by ultrasonication for 5 min. Sol–gel solution was prepared by mixing of 5 mL of TEOS, 1.5 mL of double distilled water and 100 μL of 0.1 M HCl. Using HCl in silica sol-gel can protect HRP from denaturation and accelerates formation of the sol-gel film. The GCE (diameter 3 mm) was firstly polished with alumina slurry and ultrasonically cleaned and then washed with double distilled water and acetone. Then, 10 μL of acetone/MWCNTs mixture (1.5 mg/mL) was cast on the surface of the GCE and dried in air to form a MWCNTs thin layer at GCE surface. In the next step 5 μL of HRP solution in PB was dropped on GCE/MWCNTs surface. Finally in order to trap HRP on GCE surface, 10 μL of silica sol-

gel was dropped on GCE/MWCNTs/HRP and dried at room temperature for 15 min. Finally, modified electrode washed with PB for several times to release the un-immobilized enzymes. Biosensor fabrication steps are showed in Scheme 1.

3. Results and discussion

3.1. Characterization of the modified electrode (GCE/MWCNTs/HRP/silica sol-gel)

Figure 1a displays SEM image of GCE surface after covering with a layer of MWCNTs. As it can be seen in this figure, the surface of GCE is completely covered by MWCNTs. SEM image indicates the MWCNTs have a normal structure and hegemonically dispersed on electrode surface.



Scheme 1. Schematic representation of electrode fabrication steps.

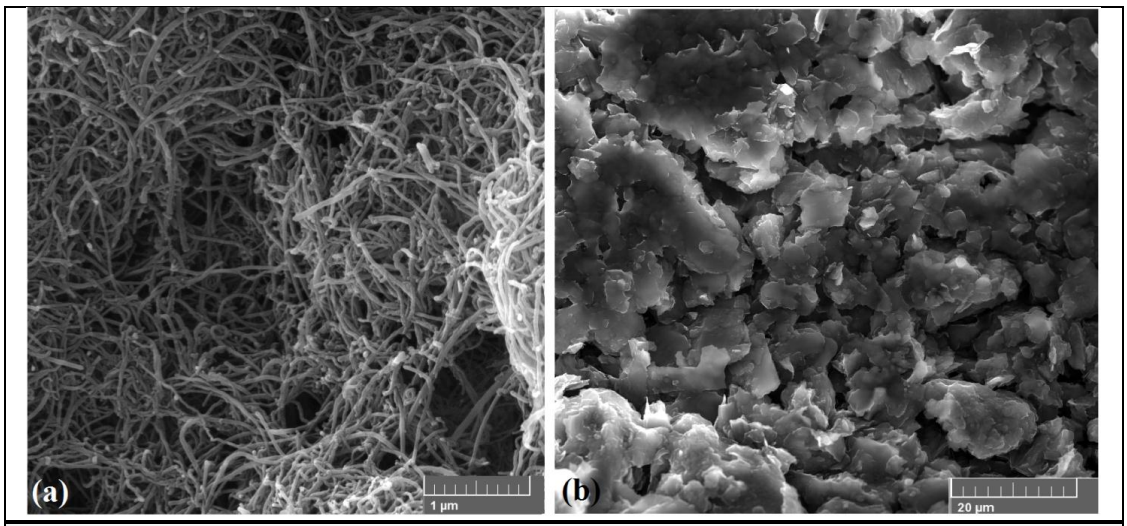


Figure 1. (a) SEM image of GCE surface modified with a layer of MWCNTs, (b) SEM image of GCE/MWCNTs/HRP/silica sol-gel surface.

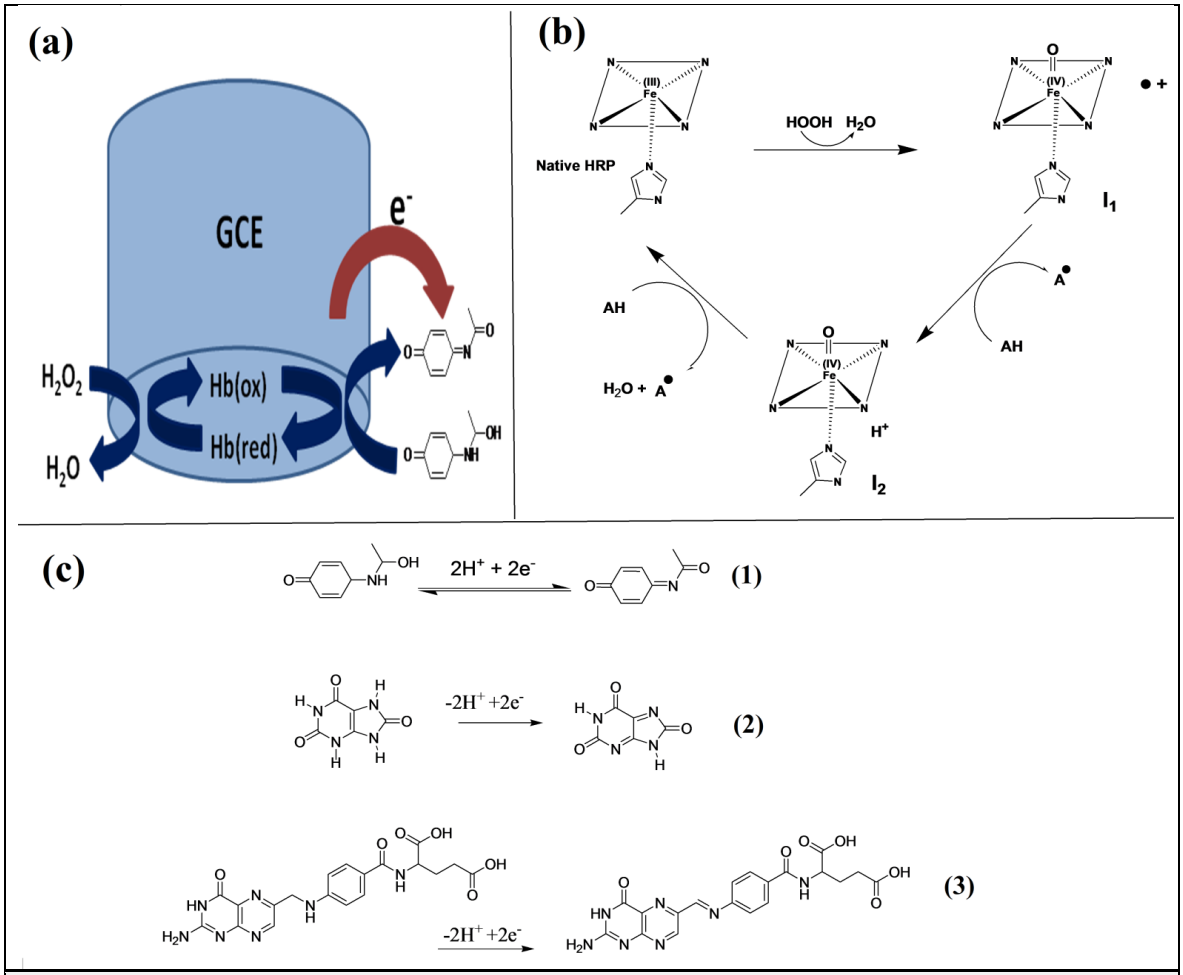
In the next step, HRP solution was dropped on GCE surface and left to get dried. Finally, to form silica sol-gel film on GCE/MWCNTs/HRP, a small drop of silica sol-gel was dropped on the electrode surface. After the formation of sol-gel film, it was washed several times with PB. Figure 1b shows the SEM image of GCE/MWCNTs/HRP/silica sol-gel surface. As can be seen in this figure, dried and layered sol-gel film has completely covered the MWCNTs/HRP content on GCE surface and consequently, has provided appropriate cover to prevent direct contact of HRP with solution.

3.2. The principle of HRP-modified electrode for acetaminophen detection

The electrochemical and redox reactions occurred during the electrocatalytic reduction of acetaminophen on GCE/MWCNTs/HRP/silica sol-gel surface in the presence of H_2O_2 is shown in S12/5/2018cheme 2a. As it is shown, first HRP (hem as prosthetic section of enzyme) is oxidized by H_2O_2 available in analyte solution and then reduces back to the first native state by oxidizing of acetaminophen, then oxidizes phenolic (quinine or an electroactive phenoxy radical) compound get reduced on the electrode surface. This results in reduction current which can be directly related to phenolic concentration in analyte solution. Oxidation of HRP by H_2O_2 and its reduction by acetaminophen are represented in detail in Scheme 2b.

As shown in this Schematic, a cycle is occurring through two consecutive stages, including two enzyme intermediates, I_1 and I_2. The first stage is splitting of H_2O_2 molecule with the simultaneous production of water and interpolation of one of the oxygen atoms of H_2O_2 into I_1. I_1 contains an oxoferryl group (Fe^{IV}=O), in which the iron is in +4 oxidation state, and a porphyrin p-cation radical. I_1 have the ability to oxidize a wide range of analyte molecules through a mechanism involving a single-electron transfer, in which the p-cation radical is first discharged, due to the formation of the second enzyme intermediate called I_2. I_2, which also contains an oxoferryl group (Fe^{IV}=O), is then reduced by a second substrate molecule (*e.g*. phenolic compounds) to the native ferric enzyme (Fe^{III}). This one-electron reduction turns ferryl iron to its ferric state, whereas the oxygen accepts two protons and forms a water molecule that is released from the heme [28].

Phenolics can act as well-known electron mediators in the HRP modified biosensor. Phenolic compounds reduce the oxidized HRP to native form and convert them to quinine or an electroactive phenoxy radical. These types can be efficiently reduced on electrode surface. Therefore, the reduction current is directly proportional to the phenol concentration in the solution [29]. The phenolic biosensor based on this mechanism was developed in



Scheme 2. **(a)** Redox and electrochemical reactions occurred during acetaminophen reduction on biosensor surface in presence of H_2O_2, **(b)** Details for HRP oxidation by H_2O_2 and HRP reduction by phenolic compounds, **(c)** Acetaminophen (1), UA (2), and FA (3) redox reactions.

the present work in which acetaminophen plays the role of electron mediator.

It was well known that the sensitivity of the peroxidase based biosensors for phenolic compounds can be limited by the high current due to direct electron transfer between the enzyme and base electrode (GCE in here) in the presence of peroxide. However, it seems that the enzyme immobilization by sol-gel prevents the direct electron transfer between the oxidized form of enzyme and the electrode surface in the present work.

Figure 2 shows the cyclic voltammograms of 50 mM of PB (pH=7) containing 50 mM of H_2O_2 with scan rate of 100 mV s^{-1} obtained by bare GCE (a), GCE/MWCNTs/silica sol-gel (b) and GCE/MWCNTs/HRP/silica sol-gel (c) as work electrodes. As clearly seen there is no significant difference between voltammograms obtained by GCE/MWCNTs/HRP/silica sol-gel and GCE/MWCNTs/silica sol-gel electrodes. It means that direct electron transfer between immobilized HRP and GCE was efficiently blocked. Blocking of direct

electron transfer is attributed to properties of silica sol-gel applied in biosensor modification [30]. This advantage makes developed electrode as a very sensitive biosensor for phenolic compounds such as acetaminophen. Large background current in voltammograms of (b) and (c) is almost observed in all electrodes modified with carbon nanotubes [31].

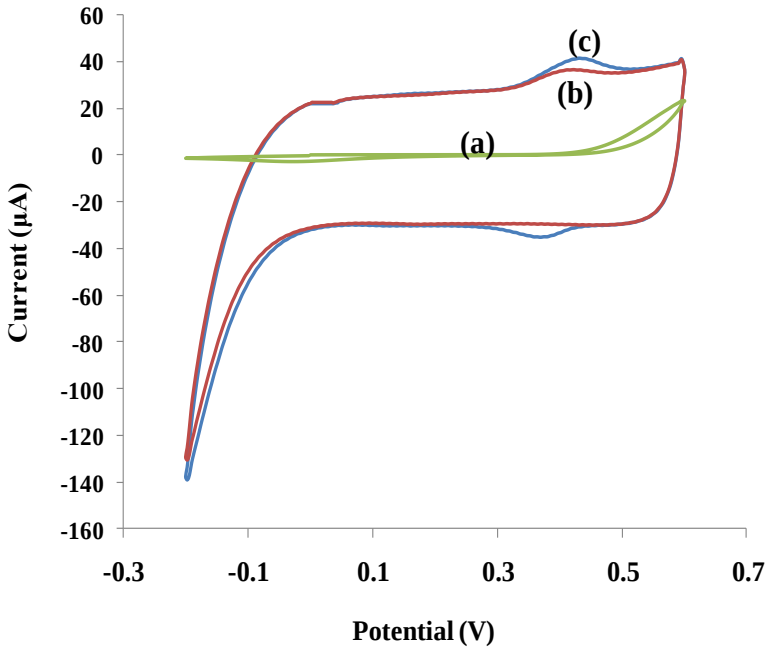


Figure 2. CVs of 50 mM H_2O_2 in PB 50 mM (pH 7) obtained by GCE (a),GCE/MWCNTs/silica sol-gel (b), GCE/MWCNTs/HRP/silica sol-gel (c) as working electrodes.

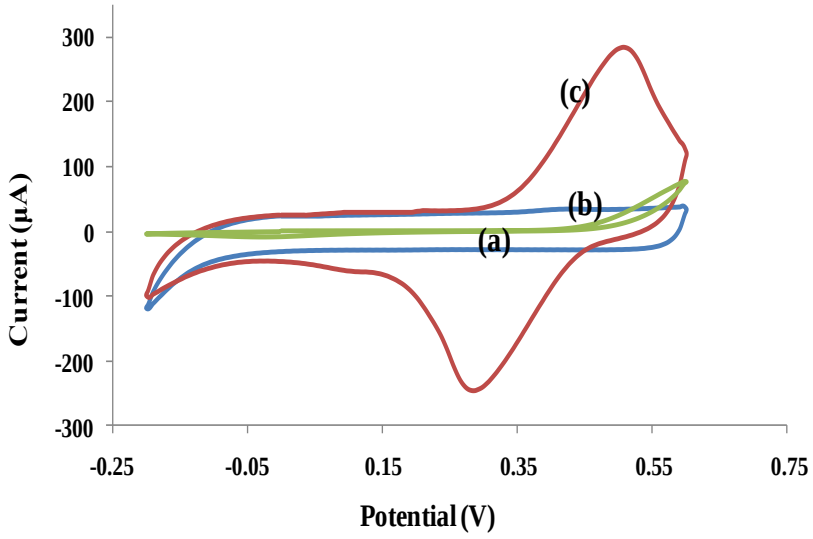


Figure 3. CVs of solution containing PB (pH 7), 1 mM of acetaminophen and 0.7 mM of H_2O_2 obtained by: GCE (a), GCE/MWCNTs/silica sol-gel (b) and GCE/MWCNTs/HRP/silica sol-gel (c).

3.3.1. Optimization of hydrogen peroxide concentration

One of the most critical parameters affecting response of HRP modified biosensors is H_2O_2 concentration. It is verified that high concentrations of H_2O_2 will reduce the enzyme activity [30]. Consequently amount of H_2O_2 is very important in achieving sensitive determination of acetaminophen. Figure 4 shows the response of biosensor to a fixed amount of acetaminophen (50 mM) in presence of different amounts of H_2O_2. As it can be seen, biosensor response is increased to 25 µM and then remains nearly constant in higher H_2O_2 concentrations. So the optimum current was regarded about 25 µM of H_2O_2. Also other experiments showed this optimum amount for H_2O_2 is approximately independent from acetaminophen concentration therefore, 25 µM of H_2O_2 was chosen for the next experiments.

3.3.2. Optimization of HRP concentration

Other parameter that affects the biosensor performance is the amount of immobilized HRP on GCE. Various volumes of HRP, 1-20 mg/mL, were used for preparation of biosensor and the corresponding voltammetric responses are shown in Figure 5. This figure shows the optimum amount of immobilized HRP which obtains the highest reduction current for fixed amount of acetaminophen. The optimized HRP amount was found to be 5 µL of HRP/PB solution of 10 mg/mL (0.05 mg HRP) and this amount was employed in all subsequent experiments. It can be observed in the Figure 5 that there is a decreasing in current after the concentration of 10 mg/mL. High amounts of HRP can prevent some of the enzyme molecules from accessing H_2O_2 because of steric hindrance, or it can decrease electronic communication at the electrode surface [32].

3.3.3. Effect of pH on biosensor response

It is well known that pH is one of the most important parameters that control the enzymatic activity and enzyme stability in aqueous media. A fixed amount of acetaminophen was determined by biosensor in pH range of 3-10. Voltammetric experiments were carried out in the batch setup. The effect of pH was investigated for the electrode in 0.1 M PB by adjusting the pH range between 3 and 10 for 1 mM of the acetaminophen. Figure 6a shows the negative shift for reduction current peaks with pH increasing in two linear ranges of 3-7 and 8-10. Negative potential shift for reduction peak currents was completely expected from redox equation for acetaminophen which is shown at Scheme 2c (1). Reduction potential vs. pH is plotted at Figure 6b. As it observed, reduction potential has been

reduced with pH in range of 3-7 by slope near to Nernst slope definition (0.0591/2), while negative shift for reduction potential occurred by lower slope in pH range of 8-11.

3.4. Comparison of the biosensor response for H_2O_2 and acetaminophen

It is known that peroxidases can catalytically reduce the H_2O_2. So it is possible that part of reduction current observed in cyclic voltammograms is related to H_2O_2 reduction in the presence of HRP. This may be strongly decreases the biosensor selectivity. Figure 7 shows the comparision of biosensor response for a

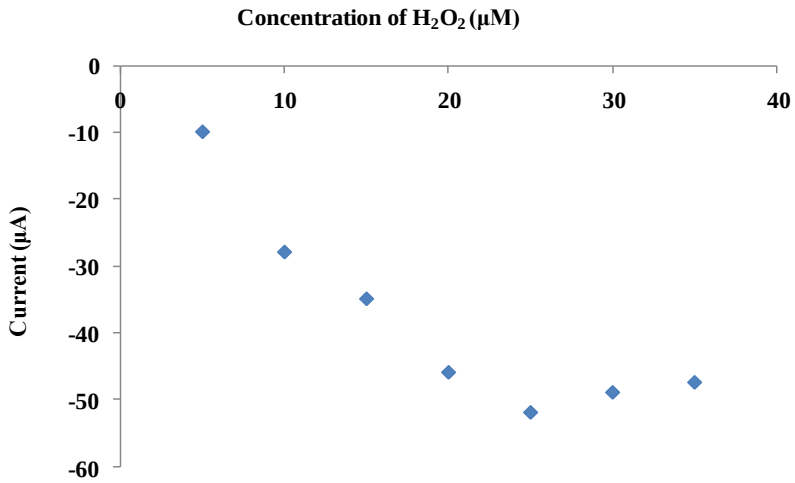


Figure 4. Biosensor response for 30 µM of acetaminophen in PB (pH 7) in presence of different amounts of H_2O_2.

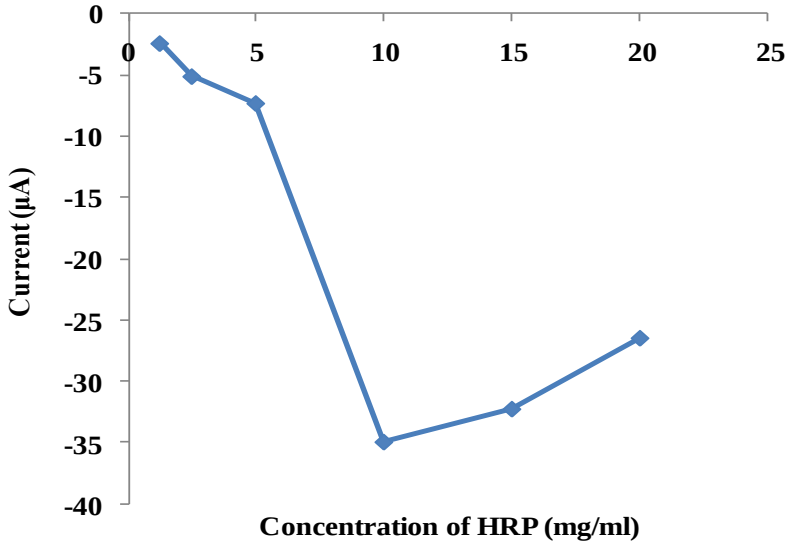


Figure 5. Effect of concentration of the HRP used in biosensor modification, on biosensor response in PB 50 mM containing 0.25 mM acetaminophen and 0.2 mM H_2O_2.

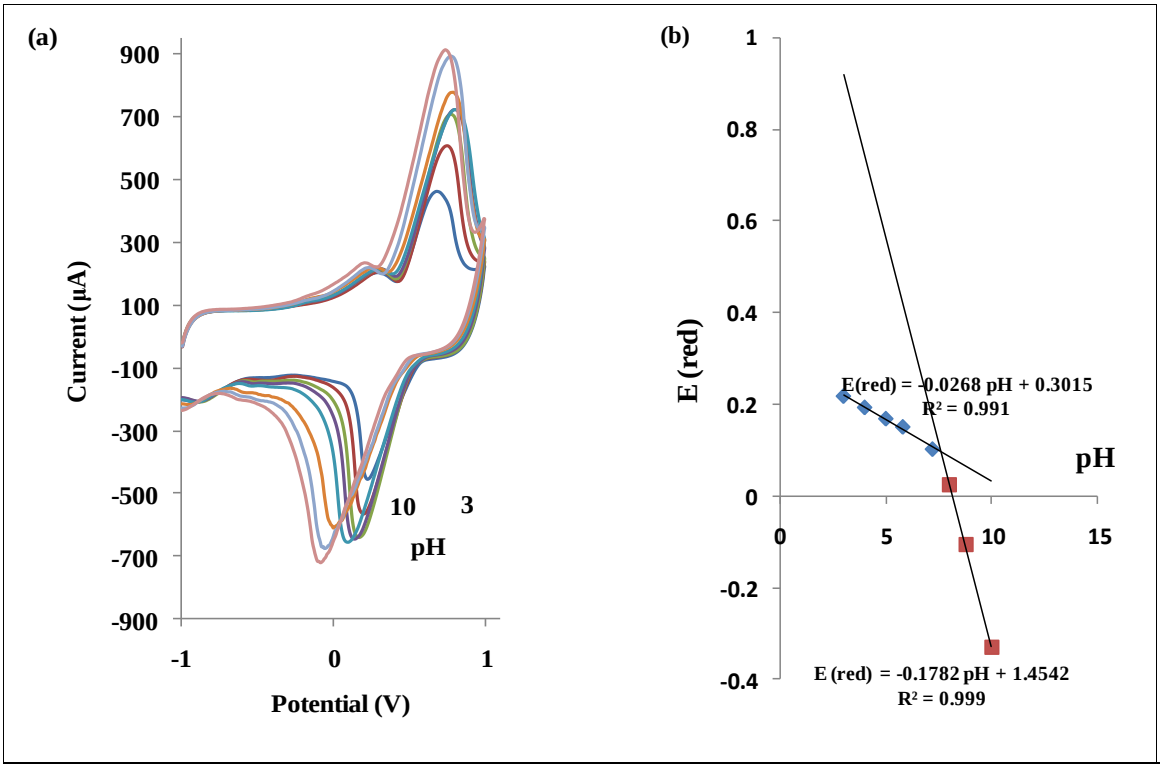


Figure 6. **(a)** CVs of acetaminophen (1 mM) obtained by biosensor at pH 3-10, **(b)** linear plots of E (red) *vs.* pH.

same amount of H_2O_2 and acetaminophen (0.7 mM) in PB in presence of a basic amount of H_2O_2 (0.5 mM). As shown in Figure 7 biosensor has almost no distinct response to H_2O_2 but strong redox peak currents for acetaminophen is observed. Accordingly developed biosensor is very selective to acetaminophen reduction in the presence of H_2O_2.

3.5. Calibration curve for acetaminophen

Figure 8a shows the cyclic voltammograms (CVs) for acetaminophen obtained by successive additions of acetaminophen to PB under the optimal experimental conditions. According to electrocatalytical reduction of acetaminophen in biosensor surface trough above mentioned mechanism, acetaminophen concentration is directly related to the reduction peak current. Therefore, calibration curve was obtained by plotting the reduction current vs. acetaminophen concentration (Figure 8b). When acetaminophen is added to the PB, the reduction current rises steeply to reach a stable value. The response time for the biosensor was completely short, reaching 90% of its maximum response in about 12 s. The calibration curve obtained for acetaminophen showed a linear response range from 1.85×10^{-6} to 2.7×10^{-3} M with a correlation coefficient of 0.999 for n=12.

This range is much larger than many other reported enzyme based biosensor for acetaminophen determination. Its detection limit was calculated about 18 nM. The equation of the regression line obtained for acetaminophen was calculated to be:

$$A = -0.221528\ C - 4.9801$$

where A is the current in microampere and C is the concentration of acetaminophen in mol/L. The sensitivity of the biosensor to acetaminophen was calculated to be 220 nA/µM. These high sensitivity and low detection limit are much better than previously reported biosensors for acetaminophen.

Performance of GCE/MWCNTs/HRP/silica sol-gel electrode was compared with some other electrode previously reported for acetaminophen determination in Table 1. As it is clear from this table, our proposed biosensor has acceptable linear range and detection limit in comparison with other reported sensors.

To evaluate the applicability of our developed electrode in acetaminophen determination in real samples, acetaminophen was measured in one sample of urine and two tablet samples by standard addition method. Urine and tablet samples were diluted with buffer and expected amount for tablet samples were calculated from reported mg of acetaminophen used in tablet and expected amount for urine sample was calculated by addition of highly Pure raw material of acetaminophen (>99%) to urine sample. Results are summarized in Table 2. Recovery is between 94.7 and 105.4% that are very satisfied for accurate drug determination in real samples.

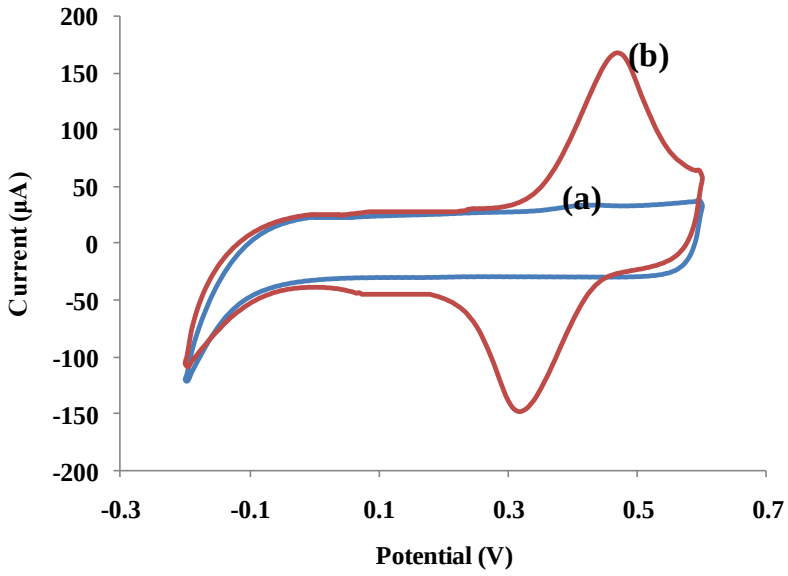


Figure 7. . Biosensor response to 0.7 mM of H_2O_2 (a) and 0.7 mM of acetaminophen (b) in presence of basic 0.5 mM H_2O_2.

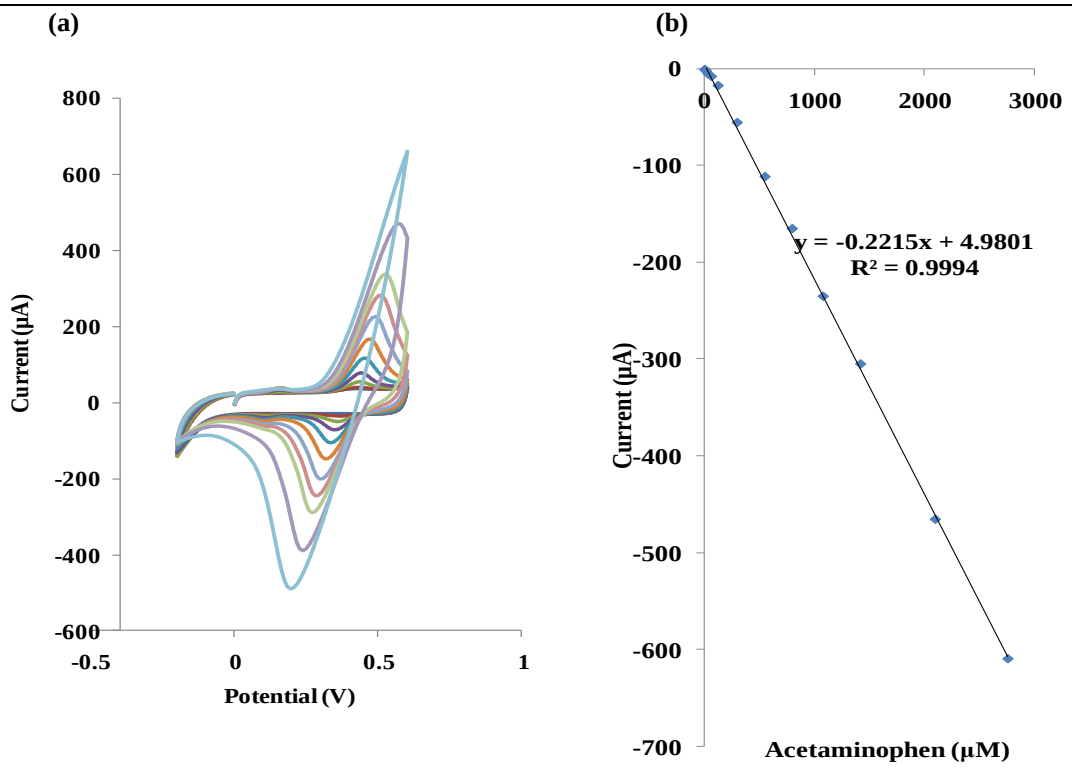


Figure 8. **(a)** CVs obtained from successive addition of acetaminophen to PB, **(b)** calibration curve for acetaminophen, resulted from cathodic peak currents of the CVs.

Table1. Comparison of our developed electrode performance with some recently reported electrodes

Method	Linear range(µM)	Detection limit (µM)	Electrode	Sensitivity (µA/µM)	Ref.
DPV[1] & CV	0.05–64.5	0.038	SWCNT[2]–GNS[3]/GCE	1.0	[37]
CV & amperometry	10–6000	1.18	(LCCH)[4]/conducting glass	0.224	[38]
CV	19.6-255	0.117	HRP/ZA[5]/GCE	0.008	[15]
DPV& CV	0.1-70	0.045	MAuNP[6]/CPE[7]	1.877	[39]
CV, DPV	8.3-145.6	1.18	AuNP-PGA[8]/SWCNT	0.01	[2]
SWV[9]	4-400	1.33	NiO–CuO/GR[10]/GCE	0.0437	[40]
CV, DPV	0.04-100	0.02	ERG[11]/Ni_2O_3–NiO/GCE	0.137	[41]
DPV & CV	0.25-50	0.025	PMR[12]/TiO_2–GR/GCE	0.463	[42]
CV	1.85-2700	0.018	Sol-Gel/HRP/MWCNT/GCE	0.599	Our work

1: differential pulse voltammetry, 2: single-walled carbon nanotube, 3: graphene nanosheet, 4: Layered cobalt carbonate hydroxide, 5: zirconium alcoxide, 6: magneto Au nanoparticles, 7: carbon paste electrode, 8: poly (glutamic acid), 9: square potential voltammetry, 10: graphene, 11: electrochemically reduced graphene, 12: poly(methyl red).

3.6. Simulataneous determination of acetaminophen, uric acid and folic acid

In addition to specific role of HRP in electrocatalytic reduction of phenolics in presence of H_2O_2, Peroxidases like HRP catalyse the oxidation of a wide variety of electron donor substrates, such as phenols, aromatic amines, thioanisoles and iodide, by H_2O_2 [33]. In this part of our research, simultaneous determination of uric acid (UA) and folic acid (FA) with acetaminophen was investigated by use of electrocatalytic oxidation of acetaminophen, UA and FA oxidation. Equations for UA and FA oxidation were shown at Schemes 2c (2) and (3) respectively.

Table 2. Determination of acetaminophen in tablet and urine samples

Sample	Added (μM)	Expected (μM)	Found (μM) (The mean of three measured values)	Recovery (%)
Tablet 1[a]	--	5.0	5.26	105.2
	5.0	10.0	10.19	101.9
	10.0	15.0	15.68	104.5
Tablet 2[b]	--	5.0	4.75	95.0
	5.0	10.0	10.54	105.4
	10.0	15.0	14.21	94.7
Urine[c]	10.0	10.0	9.69	96.9
	20.0	20.0	20.41	102.1
	30.0	30.0	29.36	97.9

a) Acetaminophen Tablet 325 mg product of Alborz Darou Co.(Iran)

b) Acetaminophen Tablet 325 mg product of Kharazmi Co.(Iran)

c) Urine sample from a 26 years old man with no taking any drug

UA and FA are the most common interferes in electrochemical measurements of drugs and biological compounds. Several studies have been reported simultaneous measurement of UA and FA with other analytes [34-36]. Therefore, simultaneous measurement of acetaminophen with UA and FA was investigated by applying the developed electrode. Results are shown in Figure 9. Differential puls voltammogramms (DPVs) of successive addition of acetaminophen, UA and FA to PB were recorded and are shown in Figure 9a and obtained calibration curves for these molecules are represented in the parts of (b) to (d)

successively. According to slopes of calibration curves, electrode is more sensitive to acetaminophen compared with UA and FA that clearly refers to electrctrocatalytic role of HRP in acetaminophen oxidation on the electrode surface. Sensitivity and linear range for acetaminophen, UA and FA in simultaneous determination are shown at Table 3.

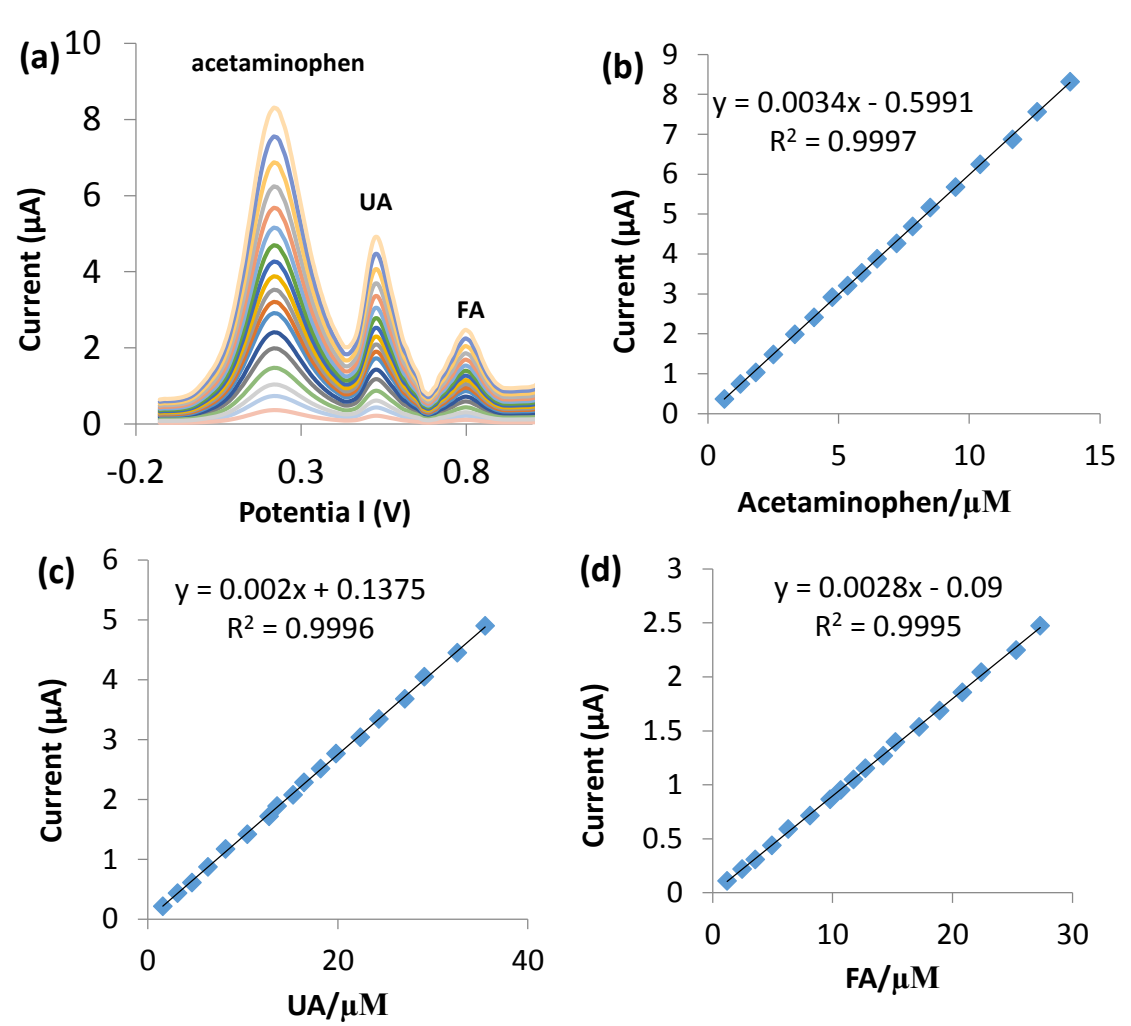


Figure 9. **(a)** DPVs of successive addition of acetaminophen, UA and FA to PB, **(b)** Calibration curve for acetaminophen, **(c)** Calibration curve for UA and **(d)** Calibration curve for FA.

Table 3. Linear range and sensitivity for acetaminophen, UA and FA in simultaneous determination.

Molecule	Sensitivity (nA/µM)	Linear range (µM)
Acetaminophen	560	0.6-14
Uric acid	140	1.5-35
Folic acid	87	1.2-27

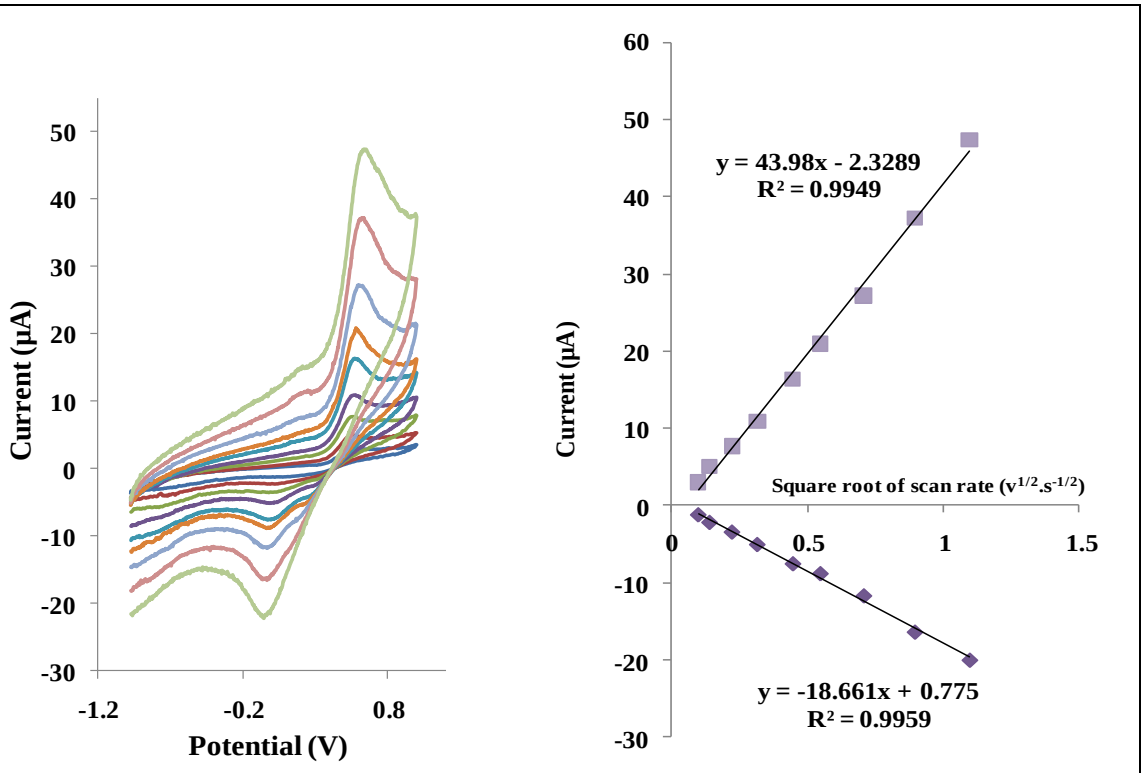


Figure 10. (a) Voltammograms of a fixed amount of acetaminophen (0.12 mM) in different potential scan rate of 10-1200 mV/S, **(b)** Cathodic and anodic peak currents *vs.* square root of potential scan rate.

3.7. Effect of potential scan rate

Figure 10a shows the voltammograms of a fixed amount of acetaminophen (120 µM) in potential scan rate of 10-1200 mV s^{-1}. As shown in Figure 10b the cathodic and anodic peak currents are linearly proportional to square root of potential scan rate ($v^{1/2}$) inferring that acetaminophen redox on biosensor surface is mainly controlled by diffusion.

3.7. Stability of biosensor

Developed biosensor was suitably stable for about 3 weeks so that, it reached 85% of primary response (response in first day of fabrication) after 3 weeks. Also it has approximately a same response after 20 successively determination of a fixed acetaminophen solution.

4. Conclusion

The developed acetaminophen biosensor based on GCE modified with MWCNTs/HRP/silica sol-gel is very sensitive, easy way to fabricate and suitably stable. Its detection limit is very low and it has linear response in a large concentration range. The sensitivity, detection limit and linear concentration range of the proposed biosensor are much better than many previously reported enzyme-based biosensors. Simultaneous determination of acetaminophen, UA and FA was successfully carried out by applying the proposed biosensor. Acetaminophen

determination in urine and tablet samples showed the good applicability of this biosensor in real samples. Finally, the fabricated modified electrode can potentially suggest a sensitive biosensor for other phenolic compounds.

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