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Screen-printed electrodes modified with HRP–zirconium alcoxide film for the development of a biosensor for acetaminophen detection

Research Article

Veronica Sima, Cecilia Cristea, Ede Bodoki, Gabriela Duțu, Robert Săndulescu*

Analytical Chemistry Department, Faculty of Pharmacy, "IULIU HAȚIEGANU" University of Medicine and Pharmacy, Cluj-Napoca 400349, Romania

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Abstract: The development of a biosensor based on the immobilization of horseradish peroxidase (HRP) within a zirconium alkoxide-polyetilenimine film onto screen-printed electrodes (SPE) for acetaminophen detection and acetaminophen quantification in pharmaceutical products is described. The biosensor operation mode is based on monitoring the amperometric signal produced by the electrochemical reduction of the enzymatically generated electroactive oxidized species of acetaminophen in the presence of hydrogen peroxide. The enzyme immobilization is performed by retention in a polyethylenimine-zirconium alcoxide porous gel film, a technique that offers good entrapping and a protective environment for the biocomponent due to the hydration properties of the immobilization layer. SPEs have the advantage of being easily mass-produced at low costs with superior characteristics in comparison with classical electrode materials. In this configuration, zirconium alkoxide demonstrates its electrocatalytic activity. The biosensor allows the quantification of acetaminophen with a limit of detection of 6.21×10^{-8} M and a linear range between 4.35×10^{-7} M and 4.98×10^{-6} M. Finally, the biosensor is applied to the quantitative analysis of acetaminophen in Perdolan® tablets.

Keywords: *Electrochemical biosensor • Horseradish peroxidase • Zirconium alkoxide • SPE • Acetaminophen*

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1. Introduction

The requirements and regulations in the fields of environmental protection, control of biotechnological processes and certification of food and water quality are becoming more and more urgent [1]. At the same time, stricter requirements regarding human and animal health have led to a rising number of clinical and veterinary tests. Therefore, there is a need to develop highly sensitive, fast and economic methods for the detection, quantification and monitoring analysis. The elaboration of biosensors is probably one of the most promising ways to approach problems concerning selective, sensitive, fast, easy to use, cost-effective and repetitive measurements with miniaturized and portable devices [2]. The major fields of practical application of amperometric biosensors are clinical diagnostics and pharmaceutical analysis. Clinical diagnostics is deliberately listed first, since more than 80% of commercial devices are used in this domain [1].

Generally, biosensors are sensitive and selective analytical devices involving the association of a biocomponent to a transducer. Besides choosing the adequate transducer and enzyme in the construction of a biosensor, an important aspect of the design is played by the method of enzyme immobilization.

The screen printed electrodes (SPE) have been developed for innovative applications in analytical chemistry. As reported in literature, the use of screen-printed electrodes (SPE) is a great simplification in the design and operation of analytical determinations, in accordance with the requirements of a decentralized assay [3]. Recently, SPEs have demonstrated promising potential, especially in the development of rapid analysis

* *E-mail: rsandulescu@umfcluj.ro*

and biosensor fabrication [4]. Screen-printed electrodes are miniaturized devices based on different layers of inks printed on an inert plastic or ceramic substrate [5]. Moreover, they are easily mass-produced at low costs [6], while retaining superior characteristics in comparison with classical electrode materials.

Horseradish peroxidase (HRP) has been a powerful tool in biomedical and pharmaceutical analysis. The wide use of HRP is due to its commercial availability [7] in a relatively pure form at reasonably low prices, its stability, and its high turnover on a variety of substrates. The list of biosensors using HRP as a biocomponent applied in biomedical and pharmaceutical analysis is very long. HRP has been applied to the detection and quantification of neurotransmitters [8], immunoassays for tumor markers [9], antigen detection [10], DNA analysis [11], the detection of carnitine [12], cholesterol [13], the antineoplasic drug ellipticine [14], glucose [15], lactate [16], clozapine [17], thiols [18], hydrogen peroxide [19], phenols [20] and ethanol [21].

The immobilization of the biocomponent should assure the entrapment of the enzyme at the surface of the electrode, while overcoming another challenge: the preservation of the microenvironment required for maintaining the enzyme's biocatalytic activity, thus ensuring the lifetime of the biosensor.

Even though the immobilization technique of the biocomponent in a matrix at the electrode's surface is a well known technique, the characteristics of the matrix can be further improved by different methods. In our case, this was done by adding zirconium alkoxide gel in order to improve the hydration of the enzyme and the analytical response of the biosensor. Lately, many approaches in the construction of biosensors use inorganic zirconium for biocomponent immobilization [22-26]. According to the literature, thin films of nano-sized zirconium gels have been used to immobilize hemoglobin [22], myoglobin [23], DNA [24], and HRP [25,26]. The analytical characteristics of the biosensors developed proved that the nanogel presented electrocatalytic activity and maintained a well-hydrated microenvironment for the enzyme. Therefore, due to its biocompatibility, good electrical conductivity and its affinity for oxygen-containing groups, ZrO_2 nanogels are ideal materials for the construction of biosensors.

In the present work, the HRP biocomponent was immobilized onto the transducer surface (the working electrode of the SPE) by entrapment into a porous zirconium alkoxide gel - polyethyleneimine film.

The resulting device was used to monitor the amperometric signal produced by the electrochemical reduction of the enzymatically-generated, electroactive oxidized species of acetaminophen (N-acetylbenzoquinoneimine - NAPQI) in the presence of hydrogen peroxide [27] (Fig. 1).

$$\text{Acetaminophen} + H_2O_2 + 2H^+ + \text{HRP} \rightarrow \text{NAPQI} + 2H_2O$$
$$\text{NAPQI} + 2e^- + 2H^+ \rightarrow \text{Acetaminophen}$$

The biosensor was tested for the assay of acetaminophen in the drug Perdolan® using the standard addition method.

2. Experimental Procedure

2.1. Chemicals

The horseradish peroxidase enzyme (1.11.1.7 type II, 180 U mg^{-1}), sodium dihydrogenphosphate, sodium monohydrogenphosphate and hydrogen peroxide 30% were provided by Sigma, acetaminophen was provided by Merck and poly(ethyleneimine) (MW 60000) (PEI) from Aldrich. All reagents were of analytical grade, used as received.

The composition of Perdolan® declared by the producer is 200 mg acetylsalicylic acid, 200 mg acetaminophen, 46 mg caffeine, talc, maize starch, microcrystalline cellulose and polyvidone acetate.

The zirconium alkoxide gel was prepared according to descriptions in the literature [28]. In order to obtain the zirconium alkoxide nanogel, an alcoholic solution of 0.25 M zirconium nitrate was refluxed for 2 hours at 90ºC. It was then mixed for another 4 hours, allowing cooling at room temperature.

The stock solutions of acetaminophen were dissolved in phosphate buffer (1.00×10^{-2} M, pH=7.4) and refrigerated.

Ten tablets of Perdolan Compositum® were precisely weighed and grinded. Then, 0.56514 g of the powder (average weight of 1 tablet) was precisely weighed and dissolved in methanol in a 100 mL calibrated flask. The solution was then filtered by a paper filter (Whatman 11.0 cm, ultra-fine filter with retention down to 0.7 µm in liquids) and the first 20 mL of solution that were filtered were discarded. 1.89 mL of the filtered solution was diluted with 0.1 M phosphate buffer (pH=7.4) to obtain a 25 mL stock solution of paracetamol from Perdolan® tablets.

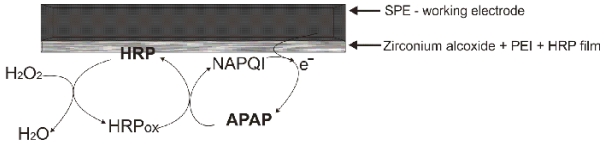

Figure 1. The mechanism of biocatalytic peroxidation and amperometric detection of acetaminophen with the HRP - zirconium alkoxide nanogel - poly(ethyleneimine) - SPE biosensor.

The screen printed electrodes (SPEs) were purchased from DropSens (Oviedo, Spain). They consisted of a graphite working electrode (d=4 mm), an auxiliary eletrode and a silver reference electrode.

The glassy carbon electrode (d=3 mm) used in the conventional three electrode cell as a working electrode was purchased from BAS Inc. (West Lafayette, USA) and it was carefully washed with demineralized water and polished with diamond paste (1 μM, BAS Inc.).

2.2. Zirconium alkoxide-PEI-ethanol-water gel preparation

5 mg PEI, 125 μL ethanol and 120 μL distillated water were mixed in an Eppendorf vial for 15 min using a vortex mixer. Next, 6.5 μL zirconium alkoxide porous nanogel, prepared as described above, were added into the initial solution and the resulting mixture was stirred for another 15 min.

2.3. Enzyme immobilization

A solution containing 0.06 mg mL^{-1} HRP in 0.1 M phosphate buffer (pH 7.4) was prepared. Equal amounts of the enzyme solution and the zirconium alkoxide-PEI-ethanol-water gel described above were mixed for 15 min. 20 μL of the resulting mixture was deposited on the working surface of the SPE by 4 successive 5 μL additions, and it was dried overnight at 4ºC. Afterwards, the gel-coated SPE was left for another 24 h at 4ºC in 5 mL phosphate buffer for hydration.

2.4. Electrochemical methods

The experiments were done with an AUTOLAB PGSTAT 30 potentiostat (Ecochemie, The Netherlands) equipped with GPES and FRA2 software.

All the cyclic voltammetry experiments were recorded with an applied potential between -0.8 V and +1.0 V *vs*. Ag quasireference electrode with a scan rate of 100 mV s^{-1}. The cyclic voltammetry determinations performed in the conventional three electrode setup used a glassy carbon electrode as the working electrode, platinum wire as the auxiliary electrode, and an Ag/AgCl 3 M KCl electrode as the reference electrode.

During amperometric experiments, the biosensor's potential was kept at -0.2 V *vs*. Ag quasireference under continuous stirring conditions. The working potential was imposed and the background current was allowed to reach a steady state value. Different amounts of acetaminophen standard solution were added every 100 seconds into the stirred electrochemical cell, and the current was recorded as a function of time.

All the experiments were performed in 0.1 M phosphate buffer (pH=7.4). The pH of the solution was adjusted using a ChemCadet pH-meter. The amperometric experiments were performed in the presence of 0.2 mM hydrogen peroxide. All tests were performed at room temperature (25ºC).

3. Results and Discussion

3.1. Film characterization

In the case of SPEs modified with 10 μL film without HRP (5 mg PEI, 125 μL ethanol and 120 μL distillated water), in control solution (phosphate buffer 0.1 M pH=7.4) at a voltage of 0.57 V *vs*. Ag quasireference, an irreversible oxidation peak is observed, which becomes much smaller at the second cycle. The compound formed during the first cycle is not further reduced, so the oxidation current at the second scanning is much smaller. It is not known which compound is oxidized, or whether this compound is part of the film, or an impurity in the film. The second cycle, which is stable for multiple scans, was taken to be the background level.

A higher background current in the control solution is observed in the case of film modified SPEs in comparison with the film modified glassy carbon electrode. This could most likely be explained by the electrocatalytic activity of the zirconium alkoxide in the case of the SPE, probably due to specific interactions between the zirconium and the electroconductive graphite-based ink used to print the SPE working electrode. Therefore, after the non-enzymatic film modification of the SPE working electrode, the appearance of spurious redox peaks is recorded, probably due to electroactive impurities present in the film.

After the deposition of the zirconium alkoxide-PEI film onto the glassy carbon working electrode, the electrochemical signal of paracetamol decreases, as a consequence of the blockage of the electroactive surface of the working electrode by the PEI (Fig. 2 and Table 1). These data are in aggrement with the results described in previous work [26], where the effect of PEI and zirconium alkoxide on the electrochemical signal of paracetamol were studied.

After the deposition of the zirconium alkoxide-PEI film onto the surface of the SPE working electrode, the electrochemical signal of paracetamol increases, probably due to the catalytic activity of the zirconium alkoxide. Another difference between the unmodified and the film-modified SPE is the peak separation voltage (ΔEp): in the case of the unmodified SPE ΔEp was 0.5 V, and in the case of the modified SPE ΔEp was 0.29 V (Fig. 2 and Table 1). The smaller difference between the oxidation and reduction potential in the case of the film-

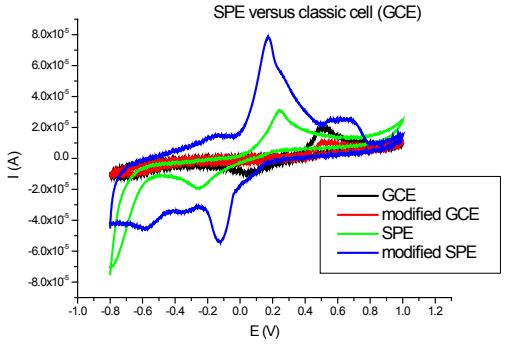

Figure 2. Classic cell with 3 electrodes and SPE - unmodified and 20 μL non enzymatic film (zirconium alkoxide-PEI-ethanol-water gel) modifed working electrode in paracetamol 6.25×10^{-4} M, 0.1 M phosphate buffer pH=7.4 – cyclic voltammetry, applied potential between -0.8 V and 1.0 V, scan rate 100 mV s^{-1}.

Table 1. Oxidation and reduction potentials and currents obtained with GCE and SPE unmodified and modified with thin film of ZrO_2–PEI in the presence of 6.250E-04 M acetaminophen solution. v = 100 mV s^{-1}, Ag/AgCl, 3M KCl reference electrode, Pt wire counter electrode.

Type of electrode		Potential (V)	Current intensity (A)
Glassy carbon unmodified electrode	Ox	+0.511	0.580E–05
	Red	-0.046	-7.229E–06
Glassy carbon modified electrode	Ox	+0.523	0.346E–05
	Red	-0.018	-1.393E–06
Unmodified SPE	Ox	+0.241	6.245E-06
	Red	-0.247	-1.272E-05
Modified SPE	Ox	+0.173	6.091E-05
	Red	-0.122	-3.075E-05

modified SPE demonstrates that the zirconium alkoxide facilitates electron transfer between the analyte and the working electrode. These data are in agreement with the literature.

There are clear differences between the classical cell and the SPE (Fig. 2). It can be concluded that the SPE has several clear advantages compared to the glassy carbon electrode. The manifestation of the catalytic capacity of the zirconium alkoxide in the case of the SPE, the improved electronic transfer between the analyte and the working electrode of the SPE, and a stronger electrochemical signal in the case of the modified SPE in comparison with the unmodified SPE results in a lower limit of detection and a higher sensitivity for the amperometric analysis of paracetamol using the HRP-SPE biosensor. The SPE also has other advantages. Even in the case of the unmodified SPE, the intensity of the oxidation and the reduction currents of paracetamol are much larger compared to the unmodified glassy carbon electrode. This is explained by the larger diameter and rougher surface of the SPE, which further increases its active surface (Table 1).

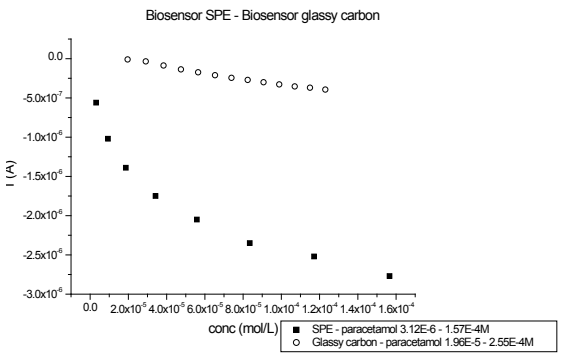

Figure 3. Comparison of the amperometric response of the HRP–ZrO_2–PEI SPE biosensor and of the HRP–ZrO_2–PEI glassy carbon electrode biosensor for paracetamol in the presence of 0.2 mM H_2O_2, 0.1 M phosphate buffer pH=7.4–amperometry, applied potential -0.2 V.

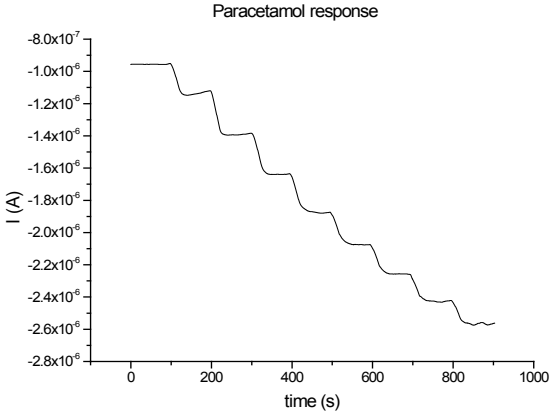

Figure 4. Amperometric response the HRP–ZrO_2–PEI–SPE biosensor for paracetamol between 4.35×10^{-7} and 4.98×10^{-6} M in the presence of 0.2 mM H_2O_2, 0.1 M phosphate buffer pH=7.4–amperometry, applied potential -0.2 V.

3.2. Amperometric testing of the biosensor and its analytical performance

The amperometric testing of the biosensor was carried out at a voltage of -0.2V *vs.* Ag quasireference, the reduction voltage of NAPQI on the modified SPE (Fig. 3).

Control experiments were performed with the zirconium alkoxide-PEI-SPE electrode (*i.e.*, without enzyme) and at the HRP-zirconium alkoxide-PEI-SPE electrode (*i.e.*, without hydrogen peroxide). Under the selected experimental conditions, no response was observed at -0.2 V for acetaminophen.

The sensitivity obtained with the SPE is much better than that obtained with the glassy carbon electrode. The two experiments were carried out using the same fabrication technique for the entire biosensor (same composition of the enzymatic film) and using the same electrochemical parameters. Even if the electrochemical

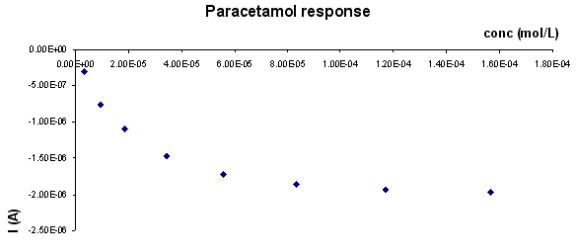

Figure 5. Michaelis–Menten curve of the HRP–ZrO_2–PEI–SPE biosensor response for paracetamol between 3.12×10^{-6} and 1.57×10^{-4} M in the presence of 0.2 mM H_2O_2, 0.1 M phosphate buffer pH=7.4 – amperometry, applied potential -0.2 V.

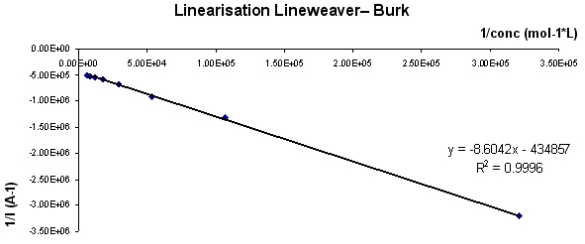

Figure 6. Linearization Lineweaver-Burk of the HRP–ZrO_2–PEI–SPE biosensor response for paracetamol between 3.12×10^{-6} and 1.57×10^{-4} M in the presence of 0.2 mM H_2O_2, 0.1 M phosphate buffer pH=7.4 – amperometry, applied potential -0.2 V.

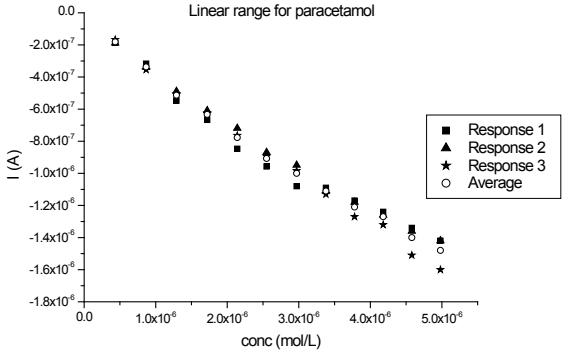

Figure 7. Linear range of the HRP–ZrO_2–PEI–SPE biosensor for acetaminophen between 4.35×10^{-7} and 4.98×10^{-6} M in the presence of 0.2 mM H_2O_2, 0.1 M phosphate buffer pH=7.4 – amperometry, applied potential -0.2 V, current measured 100 s after addition of acetaminophen.

active surface area of the SPE is larger than that of the GCE, the difference of sensitivity between the two electrodes is much larger than what this difference can account for. This leads us to believe that the improved sensitivity is due to factors other than the larger active area. Fig. 4 shows the typical amperometric response for paracetamol in the concentration range 4.35×10^{-7} to 4.98×10^{-6} M obtained with the HRP–ZrO_2–PEI–SPE biosensor.

Fig. 5 presents the typical Michaelis-Menten curve of the biosensor response. The linearized Lineweaver-Burk plot can be seen in Fig. 6. As shown, the biosensor response follows the kinetics of a typical enzymatic process. I_{max} corresponds to the maximum rate achieved when the enzyme is saturated by the substrate. Consequently, it is proportional to the total amount of active enzyme immobilized within the film. The K_M value is the substrate concentration at which the reaction rate reaches half of its maximum value. The apparent K_M is 1.98×10^{-5} M and I_{max} is -2.30×10^{-6} A.

The limit of detection (LOD) and quantification (LOQ) were calculated as the ratio of the standard deviation of the base line of the control solution (0.1 M phosphate buffer pH=7.4), which was considered the noise, and the biosensor's response to paracetamol. The establishment of LOD=6.21×10^{-8} M paracetamol (RSD=10.8%) was based on a ratio of 3 and of LOQ=2.07×10^{-7} M paracetamol (RSD=10.8%) was based on a ratio of 10.

The HRP–ZrO_2–PEI–SPE biosensor's linear trend of current versus paracetamol concentration was found to be between 4.35×10^{-7} and 4.98×10^{-6} M in the presence of 0.2 mM H_2O_2 in 0.1 M phosphate buffer pH=7.4 ($y=-0.2812\times10^{-7}$, R^2=0.990, RSD of slope=8.27%, n=3) (Fig. 7). Because of the good reproducibility of the amperometric signal in this concentration range, we used this interval for the tests of the concentration of paracetamol in the pharmaceutical forms, the Perdolan® tablet in our case.

The amperometric response for a larger concentration interval of paracetamol was tested in terms of intra- and inter-day reproducibility (Fig. 8). When not in use, the biosensor was stored in 0.1 M phosphate buffer, pH=7.4 at 4ºC. During the first test of the day, the amperometric signal was always larger in comparison with the last two tests of the day, which always showed a good reproducibility (the R.S.D.s of the slopes of the linearized responses by the Lineweaver-Burk method were less than 15%). In order to determine if this phenomenon is due to a possible structural modification of the film by the analyte or by the product of the enzymatic reaction, or whether it is due to the modification of the potential of the quasireference electrode during storage in phosphate buffer, experiments with an external reference Ag/AgCl (3 M KCl) electrode were carried out. In the case of the external reference, there were no differences between the first and subsequent experiments. We can conclude that modifications of the surface of the quasireference electrode occurs during storage, and its potential changes as a result. After one determination, equilibrium is probably reached, which is why the next determinations show good reproducibility. Therefore, the replacement of the phosphate buffer with

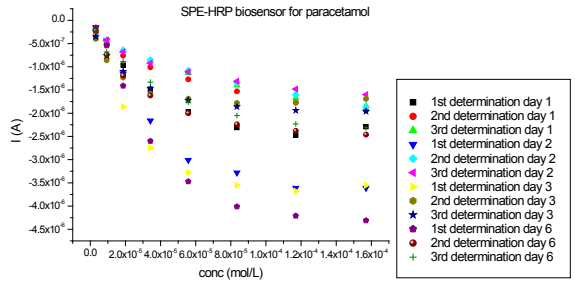

Figure 8. Amperometric responses of the HRP–ZrO_2–PEI–SPE biosensor as a function of acetaminophen concentration between 3.12×10^{-6} and 1.57×10^{-4} M along 5 days in the presence of 0.2 mM H_2O_2, 0.1 M phosphate buffer, pH=7.4 – amperometry, applied potential -0.2 V, current measured 100 s after addition of acetaminophen.

a chloride-containing buffer is recommended to stabilize the potential during the first determination.

From one day to the next, the signal constantly increased, probably due to better hydration and re-arrangement of the film on the surface of the SPE. The biosensor was not tested for a longer period because this was not considered of interest. SPEs are single-use devices, and keeping them in phosphate buffer is not advisable due to the possible modifications of the quasi-reference electrode.

3.3 Acetaminophen tablet assay

The biosensor was applied for the assay of acetaminophen in Perdolan® tablets. The standard addition method was used by adding approximately equal aliquots of acetaminophen, 6.23×10^{-7} M, from the Perdolan® formulation and from a standard solution in 0.1 M phosphate buffer (pH 7.4) in the presence of 0.2 mM H_2O_2.

The results obtained (198.86±4.19 mg/tablet, n=5) are consistent with those obtained with the HRP-NMPs (nanomagnetic particles) biosensor (198.0±0.8 mg/tablet, n=9) [27] and with the amount declared by the manufacturer (200 mg/tablet).

To our knowledge, no SPE-HRP biosensors for acetaminophen quantification in pharmaceutical formulations have been described in the literature. If we compare the performances of the biosensor described herein with one of our devices presented in previous work [26], where the same immobilization method of the biocomponent was used on a glassy carbon electrode, this new SPE-HRP biosensor presents a lower limit of detection (6.21×10^{-8} M in comparison with 1.17×10^{-7} M) and also a higher sensitivity. Compared to an on-line microfluidic sensing device described by Messina *et al.* [29], the performances in terms of limit of detection and linearity range are quite similar. Both the on-line microreactor and our device, which respects the principles of biosensors (the biocomponent being in close proximity with the transducer element), allow the quantification of paracetamol in pharmaceutical forms with reasonable accuracy.

4. Conclusion

An HRP-zirconium alcoxide porous gel - SPE biosensor for acetaminophen determination has been realized. The enzymatically generated electroactive oxidized species of acetaminophen in the presence of hydrogen peroxide were electrochemically reduced, and the amperometric signal was recorded.

The LOD of this method for acetaminophen is 6.21×10^{-8} M and the linear range is between 4.35×10^{-7} M and 4.98×10^{-6} M.

The zirconium alkoxide-PEI porous gel investigated represents not only a method of biocomponent immobilization onto the SPE, but it also creates a hydrated, protective and nontoxic environment for HRP, important in maintaining and preserving the enzyme's bioactivity.

The cyclic voltammetry assays showed that SPEs have clear advantages compared to glassy carbon electrodes. The electrochemical signal for paracetamol is larger in the case of unmodified SPE in comparison with the unmodified glassy carbon electrode due to the difference of the active surface area of the working electrodes. Moreover, when using film modified electrodes, in the case of the SPE the zirconium alkoxide-PEI porous gel demonstrates its electrocatalytic activity and facilitates the electron transfer. Therefore, after modification of the SPE surface, the electrochemical signal for paracetamol increases, contrary to the glassy carbon electrode where it decreases. After film deposition, the ΔEp decreases in the case of SPE and remains unchanged for the glassy carbon electrode.

Taking in consideration the simplicity of fabrication of this HRP-zirconium alkoxide-PEI porous gel-SPE biosensor, it may represent an interesting analytical device for investigation of other compounds that can be peroxidated, as well as for inhibitors of the enzymatic system.

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