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Electrochemical detection in vitro and electron transfer mechanism of testosterone using a modified electrode with a cobalt oxide film

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1. Introduction

Testosterone (4-androsten-17ß-ol-3-one) (Fig. 1) is a steroid hormone from the androgen group that plays important roles in male sexual differentiation, protein synthesis and human physical performance [1]. This compound and other anabolic androgenic steroids (AAS) have been used by athletes since the 1950s as doping substances to increase muscle mass. The World Anti-Doping Agency (WADA) prohibited their use to ensure fair play and protect athletes from possible adverse side effects [2]. Chromatographic methods including HPLC [3], GC–MS [4] and LC–MS [5] were developed for the detection and quantitation of testosterone in biological fluids. These analytical methods provide quantitative and confirmatory results with high sensitivity and selectivity, but require some expensive instrumentation and suffer from considerable time delays between sampling and obtaining the results. These disadvantages limit their daily routine use in laboratory environments [6]. Electrochemical techniques using modified electrodes have proved to be a sensitive and versatile method for the determination of analytes undergoing biologically important reactions of oxidation and/or reduction, including isolated drugs and related molecules in pharmaceutical dosage forms. Due to their extreme simplicity, low cost, relatively short analysis time, high sensitivity and specificity compared to other techniques, electroanalytical techniques have arisen as a stand out in the field of steroid analysis [7–12]. However, some difficulties have arisen in modifying electrodes in order to analyze steroid hormones because they are small, rigid, hydrophobic molecules, and possess few functional groups for specific interaction with antibodies.

The electrochemical method presented in this work is a promising substitute for the frequently reported chromatographic methods due to its simplicity, rapidity, reliability and low cost of analysis. In recent years, the application of modified electrodes has been found to enhance the sensitivity of electrochemical determinations. In this work, a new electrochemical sensor for the detection of testosterone in vitro using a cobalt oxide-modified edge plane glassy carbon electrode is described. The preparation of a modified electrode combining the immobilization methods of cobalt
oxide on the glassy carbon electrode (GCE), works as a sensitive electrochemical transducer of testosterone that constitutes a challenge.

2. Experimental

2.1. Apparatus and electrodes

The electrochemical experiments were performed using an AUTOLAB PGSTAT302N electrochemical analyzer driven with NOVA 1.10 software. A glassy carbon electrode as a working electrode (BAS Model MF 2012; geometric area = 0.0774 cm²), Hg/Hg₂Cl₂/KCl (sat.) (SCE) as reference electrode, a platinum plate as auxiliary electrode and a three-electrode standard cell with a capacity of 50 mL were used in the experiments.

2.2. Reagents and solutions

A solution of 20 mM Co(NO₃)₂·6H₂O (Sigma–Aldrich) and 0.10 M Na₂SO₄ (Sigma–Aldrich) was prepared with double distilled water. A solution of 0.10 M NaOH (Merck) was prepared and its pH was adjusted to 12.5 with the addition of several drops of 0.010 M HNO₃ (Merck). Testosterone (Fluka, >99%) was purchased from Biospacific. A stock solution of testosterone (1.0 mM) was prepared in methanol/water (1:1) (methanol from Merck, 99%). Specific amounts of the stock solution of testosterone were added into an electrochemical cell containing the support electrolyte (0.10 M NaOH).

A calibration curve based on the values of cathodic peak current from the reduction of Co⁴⁺/Co³⁺ species was obtained from the cyclic voltammograms. All measurements were performed in triplicate. Subsequently, the detection limits (LOD) were calculated at a significance level of 95% (p < 0.05). The LOD values corresponded to three times the standard deviation of the blank current signal, calculated from 21 measurements divided by the slope of the calibration curve. Blank signals were measured from the modified electrode with cobalt oxide in 0.10 M NaOH alone in the absence of testosterone.

2.3. Cobalt film preparation

Prior to modification, a glassy carbon electrode (GCE) was pretreated in two ways in order to obtain reproducible results: (1) mechanical polishing on a micro-cloth pad with a 0.05 μm α-alumina suspension and (2) electrochemical treatment by applying a potential of 1.5 V for 4 s in the same solution in which the measurements were carried out. The electrode cleaning procedures were carried out for each experiment and this pretreatment required 5 min. All experiments were performed at room temperature.

Cobalt oxide films were deposited in a cyclical voltage process at 50 mV s⁻¹ between −1.0 and 1.0 V vs. SCE. The depositions were performed in an aerated environment of 20 mM Co(NO₃)₂·6H₂O containing 0.10 M Na₂SO₄ solution. The electrodeposited cobalt oxide films were removed from the glassy carbon electrode by polishing the surface with a 0.05 μm α-alumina suspension on a polishing micro-cloth and washing with double distilled water.

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The nominal surface concentration of the cobalt oxide film electrodedeposited on the glassy carbon surface was evaluated by cyclic voltammetry in aqueous 0.10 M NaOH and a 100 mV s⁻¹ scan rate between −1.0 and 0.5 V vs. SCE.

3. Results and discussion

3.1. Film deposition

Cobalt oxide films can be deposited electrochemically under anodic conditions onto a number of different conducting substrates such as glassy carbon, gold, platinum, transparent ITO electrodes, etc. Typical cyclic voltammograms of cobalt oxyhydroxide have growth at 50 mV s⁻¹ between −1.0 and +1.0 V vs. SCE in a 20 mM Co(NO₃)₂·6H₂O solution containing 0.10 M Na₂SO₄, examples of which are shown in Fig. 2. As can be seen, the bulk deposition of cobalt species increases at a constant rate. The maximum rate of cobalt deposition was observed only at high potentials (i.e. 0.9/1.1 V), where the formation of Co⁴⁺ species on the electrode surface is accompanied by simultaneous massive oxygen gas evolution. In fact, it has already been stated that oxygen evolution generally increases at significant rates only when potentials corresponding to higher oxidation states of the cobalt oxide are attained.

The variations in the curve profiles of the voltammograms shown in Fig. 2 during continuous cycling of the potential seem rather complex, indicating that the cobalt deposition process involves multiple simultaneous electrochemical processes on the electrode surface. The initial process involves the oxidation of Co⁰ ions to metallic cobalt (Co⁰). With an increase in the number of cycles, there is a gradual decrease in this cathodic peak at −1.0 V (peak a) in the first scan is related to the reduction reaction of Co³⁺ ions to metallic cobalt (Co⁰). With an increase in the number of cycles, there is a gradual decrease in this cathodic peak at −1.0 V, indicating that all the Co³⁺ ions are being slowly reduced on the layer of deposited cobalt oxide on the GCE surface. Moreover, the appearance of a small peak in the range of −0.30 to −0.10 V (peak b) can be noted, which is related to the dissolution of the cobalt layer deposited on the electrode surface [13].

The formation of the first Co³⁺ and Co⁴⁺ oxide species monolayers induces a noticeable increase in the anodic current related to
Co(OH)$_2$, but the anodic peak of the Co(OH)$_2$/Co$_3$O$_4$ pair (Eq. (1)) was not observed in Fig. 3. The absence of this signal is associated with the formation of different species of cobalt oxide on the surface of the working electrode.

According to the discussion, the voltammetric profile observed is characteristic of the cobalt species adsorbed on the surface electrode, and not the supporting electrolyte (0.10 M NaOH). Therefore, these results are associated with the formation of different species of cobalt oxide on the surface of the working electrode.

Changes in the electrochemical activity of the GCE/CoOx film at different scan rates are an important tool for elucidating the mechanism governing the redox processes of the material. To determine this, voltammograms were recorded for the modified electrode in an aqueous NaOH solution (pH 12.5) by varying the scan rate from 25 to 300 mV s$^{-1}$ (Fig. 4).

Fig. 4 shows the current values for the anodic (1) and cathodic (3 and 4) peaks showed a linear relationship with the increase in the scan rate from 25 to 300 mV s$^{-1}$, with correlation coefficients ($r^2$) of 0.9942, 0.9934 and 0.9956, respectively. These proportional increases indicate that the electrochemical process was controlled by charge transfer through the redox centers and the neighboring GCE surface. The electron transfer process is a consequence of the formation of the chemical species on the GCE surface.

However, the peak potential values were influenced by the increase in the scan rate because the cyclic voltammograms (Fig. 4) yielded a greater peak-to-peak separation. This large peak-to-peak separation is related to the slow diffusion of hydroxide ions (OH$^-$) on the electrode surface, meaning there is a limitation in the charge transfer kinetic. This limitation is due to factors such as interactions between ions from the support electrolyte and the deposited film layer, electrostatic factors, side interactions of redox pairs on the electrode surface and/or non-equivalence of the active sites present in the deposited layer.

3.2. Determination of testosterone

Cyclic voltammetry is a useful electrochemical technique for the measurement of electrode reaction kinetics. In order to test the electrocatalytic behavior of the GCE/CoOx electrode, cyclic voltammograms were recorded in aqueous 0.10 M NaOH (pH 12.5)
containing different concentrations of testosterone from 0.33 to 2.00 μM at 100 mV s\(^{-1}\) (Fig. 5).

As shown in Fig. 5, the cathodic peak currents (c and d) related to the species Co\(^{IV}/\)Co\(^{III}\) and Co\(^{III}/\)Co\(^{IV}\), respectively, experienced an intensity decrease in the presence of testosterone. This effect has been more highlighted for the reduction peak characteristic of the Co\(^{IV}/\)Co\(^{III}\) couplet. However, an increase in the intensity of the anodic peak currents from conversion between the Co\(^{III}/\)Co\(^{IV}\) couplet was also observed in the presence of testosterone (peak b). This would then be an indication that testosterone has been oxidized by the Co\(^{IV}\) species formed. Several scientific papers have evoked this mechanism in order to explain this behavior in carbohydrate oxidation, hydrazine, paracetamol and acetylsalicylic acid [15–17].

Given the above, from the immobilized CoOx layer on the GCE surface, species of Co\(^{III}\) formed (Eq. (1), peak a) were oxidized to Co\(^{IV}\) (Eq. (3), peak b). Thus, the existence of the Co\(^{IV}\) redox center was responsible for the oxidation of testosterone and regeneration of Co\(^{III}\) ions. In other words, the Co\(^{IV}\) redox center performed functions in the oxidation of testosterone, promoting oxidation of the Co\(^{III}\) species, which in turn induced an increase in the intensity of anodic current resulting from the conversion between the Co\(^{III}/\)Co\(^{IV}\) redox centers. Thus, for convenience we chose peak c in the other studies, since this peak (characteristic of the reduction of the Co\(^{IV}\) redox center) showed better resolution and sensitivity. However, species in solution, mainly organic compounds, react with electroactive species adsorbed on the electrode surface, and a chemical reaction can occur (chemical step) in the reaction medium. Generally, this step involves the formation of an ion-radical and/or a reaction between an adsorbed species and organic compounds in solution. In the first case, there is direct electron transfer between the electrode and the organic species to form ion radicals, mainly anion radicals. However, the formed radicals are usually unstable and exist only while they are adsorbed on the electrode surface [18].

The linear correlation coefficient \(r^2\) obtained for testosterone analysis with the GCE/CoOx electrode had a value greater than 0.996, which demonstrates a good linearity of the calibration curve of increasing amounts of testosterone (0.33–2.00 μM) in 0.10 M NaOH (pH 12.5) electrolyte. Scan rate: 100 mV s\(^{-1}\). The value found for the detection limit was 0.16 μM, which demonstrates that the developed electrode is sensitive enough to detect testosterone over the linear range of 0.33–2.00 μM, within a relative standard deviation (R.S.D) ranging from 1.33 × 10\(^{-5}\) to 4.00 × 10\(^{-4}\)% at a significance level of 95% (\(p < 0.05\)). These results therefore show that the presence of the cobalt oxide layer on the GCE surface is a useful tool for the detection of testosterone at low concentrations.

To prove the reliability of data obtained, the results deduced from the voltammetric method were compared with HPLC analysis of the literature [3] already cited (as listed in Table 1). As we can see clearly the data obtained indicated that the results obtained by both the methods are in good agreement.

Considering these factors, it a mechanism has been proposed (Fig. 7) which explains the decrease in the current signal for peak c (cathodic) related to the reduction of Co\(^{IV}/\)Co\(^{III}\) redox centers. In the absence of testosterone, only the charge transfer process (electrochemistry step) between the cobalt species is involved. However, the presence of the steroid suggests the formation of an anion-radical from the carbonyl group (C=O) in the steroid structure. This anion-radical is formed by capturing an electron from the oxidation of Co\(^{III}\) to Co\(^{IV}\). This electron would be responsible for the conversion of Co\(^{IV}\) to Co\(^{III}\) when the scanning is shifted to the negative direction. However, the presence of testosterone in the medium prevents the reduction of the Co\(^{IV}\) redox center on the electrode surface, resulting in a decrease in the peak current signal c.

In such a case, a second phase would be expected: protonation of the anion radical to convert the carbonyl group to an alcohol function. However, possibly this step possibly does not occur, since the reaction medium is very alkaline (i.e. the presence of H\(^{+}\) ions is very low). Moreover, in accordance with Grimshaw [19], and Lund and Hammerich [20] the mechanism of formation of the anion radical (chemistry step) is a slow process (unlike the electrochemical step) and reversible. This justifies the reduced current levels for peak c, which begin to disappear with increasing amounts of steroid in the reaction medium. The decrease in this peak is due to the formation of the intermediate anion radical, which interacts with the Co\(^{IV}\)

![Image](74x590 to 325x785)

![Image](344x594 to 594x785)

Table 1

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Testosterone determined by</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Voltammetry(^a)</td>
</tr>
<tr>
<td>LOD (μM)</td>
<td>0.16</td>
</tr>
<tr>
<td>(r^2)</td>
<td>0.996</td>
</tr>
<tr>
<td>Calibration range (μM)</td>
<td>0.33–2.00</td>
</tr>
</tbody>
</table>

\(^a\) This paper.

\(^b\) Ref. [3].
ions in the electrode surface, thus suppressing thus reduction of CoIV ions. Thus, at low scan rates the electron transfer at the electrode surface was dependent on the rate of the reaction that occurs between the oxide layer and the steroid molecule.

Conversely, at higher scan rates suppression (disappearance) of peak c was not observed, demonstrating that the electrochemical step is faster. This shows that the formation of anion-radicals from the group carbonyl (C=O) of the steroid is governed by the rate of the chemical reaction with the oxide layer. In this case, the electron transfer between the adsorbed species on the electrode surface is so fast that the chemical step (slow) does not have time to occur; thus not influencing the voltammetric profile of the modified electrode (GCE/CoOx), especially between the CoIV and CoIII redox centers [18].

3.3. Stability and reproducibility of the modified electrode

The stability of the GCE/CoOx electrode for determination of testosterone was examined by measuring the current response at a fixed concentration (0.33 M) in 0.10 M NaOH at pH 12.5. The modified electrode was used daily and stored in air. The modified electrode showed a deviation of 3.25% in the peak currents of testosterone within a week. This suggests that the electrode process presents sufficiently good stability.

The inter- and intra-day reproducibility of the GCE/CoOx was also evaluated. An R.S.D of 1.42% was obtained at the modified electrode for testosterone when electrode performance was monitored for ten consecutive days at the same concentration of the analyte (0.33 μM). The R.S.D was found to be 3.26% for testosterone.

Finally, the GCE/CoOx electrode exhibits good stability and reproducibility for the detection of testosterone.

4. Conclusions

This work describes an extremely sensitive electroanalytical procedure for the detection of testosterone based on cyclic voltammetry using a modified electrode with cobalt oxide. The modified electrode showed a stable and reproducible response toward the detection of testosterone. A mechanism has been proposed, suggests the formation of an anion-radical from the carbonyl group (C=O) in the steroid structure.

This developed approach will be a potential complement to the current methods due to its low detection limit, high sensitivity and low cost (compared with HPLC). The results show that the present method is simple, practical, and reliable and can be applied in the future for the quantification of testosterone in athletes at sports competitions.

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References


Biographies

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Valtencir Zucolotto, received his Materials Engineer degree from Federal University of São Carlos (UFSCar), Brazil, in 1997 and Ph.D. degree in Science and Materials Engineer from University of São Paulo, Brazil, in 2003. He is currently a Professor in Physical Institute of São Carlos (UFSCar), Brazil. He is the leader of Nanomedicine and Nanotoxicology Laboratory (UFSCar). He has experience in nanotechnology, development and application of nanomaterials in medicine, acting on the following topics: nanomedicine, polymers, self-assembly, grades of surface relief, nanocomposites, sensors & biosensors.

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