



Enzyme biosensor for androsterone based on 3 α -hydroxysteroid dehydrogenase immobilized onto a carbon nanotubes/ionic liquid/NAD⁺ composite electrode

R.A. Mundaca, M. Moreno-Guzmán, M. Eguílaz, P. Yáñez-Sedeño*, J.M. Pingarrón

Department of Analytical Chemistry, Faculty of Chemistry, University Complutense of Madrid, 28040 Madrid, Spain

ARTICLE INFO

Article history:

Received 21 March 2012

Received in revised form

28 June 2012

Accepted 5 July 2012

Available online 16 July 2012

Keywords:

Androsterone

Carbon nanotubes

Ionic liquid

NADH

3 α -Hydroxysteroid dehydrogenase

Electrochemical biosensor

Human serum

ABSTRACT

A 3 α -hydroxysteroid biosensor for androsterone determination has been prepared by immobilizing the enzyme 3 α -hydroxysteroid dehydrogenase (3 α -HSD) in a composite electrode platform constituted of a mixture of multi-walled carbon nanotubes (MWCNTs), octylpyridinium hexafluorophosphate (OPPF₆) ionic liquid and NAD⁺ cofactor. This configuration allowed the fast, sensitive and stable electrochemical detection of the NADH generated in the enzyme reaction. All the experimental variables involved in the preparation and performance of the enzyme biosensor were optimized. Amperometry in stirred solutions at +400 mV provided a linear calibration plot for androsterone in the 0.5–10 μ M concentration range with a slope value more than 200-times higher than that previously reported. The detection limit achieved was 0.15 μ M and a low value of the apparent Michaelis–Menten constant (K_{app}^M), 36.0 μ M, similar to that reported for the enzyme in solution, was calculated. The 3 α -HSD/MWCNTs/OPPF₆/NAD⁺ biosensor provided good results in the determination of androsterone in spiked human serum samples.

© 2012 Elsevier B.V. All rights reserved.

1. Introduction

Androsterone, 3 α -hydroxy-5 α -androstan-17-one, is an intermediate product in the synthesis of androgens in humans. It is a metabolite of testosterone as the product of a reaction catalyzed by the enzyme 17 β -hydroxysteroid dehydrogenase 17 β -HSD, but it can also be formed from other adrenal androgens such as dehydroepiandrosterone (DHEA) [1]. High levels of androsterone glucuronide are found in the human prostate, breast cyst fluid and ovary follicular fluid. Androsterone is active in the central nervous system and interacts with the gamma-aminobutyric acid (GABA), being considered a neuroactive steroid [2]. It also has a weak activity in regulating male sexual characteristics, and so, it is used in veterinary medicine to counter the effects of castration as well as in bodybuilding activities to increase muscle mass. In this sense, it is considered as an anabolic androgenic steroid, a kind of doping substances whose use was first forbidden by the International Olympic Committee (IOC) at the Olympic Games, held in Montreal, Canada in 1976. The detection of androgenic, anabolic steroids, either from endogenous or synthetic origin, is one of the major tasks in doping analysis. Much work has been done in the last decades to develop analytical methodologies for the detection

of doping substances in biological samples [3]. Recent examples involve the use of gas chromatography–tandem mass spectrometry (GC–MS/MS) for the analysis of mixtures of androsterone and other androgenic and estrogenic hormones [4,5]. Gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) was also applied for steroid confirmation analysis in real time during competition [6]. Moreover, the determination of anabolic steroids has also been performed by LC–MS [7,8].

Hydroxysteroid dehydrogenases are a family of nicotinamide nucleotide-dependent oxidoreductases that catalyze interconversions of hydroxy and carbonyl groups of steroids in a positional and sterically specific manner [9]. One of these enzymes, 3 α -HSD, catalyzes specifically the oxidation of the hydroxyl group in position 3 of the molecule. The development of enzyme biosensors based on 3 α -HSD constitutes an interesting analytical strategy for the selective detection of 3 α -hydroxysteroids in complex samples. However, this alternative has been scarcely exploited, and a very few 3 α -HSD biosensors have been described in the literature. Regarding electrochemical biosensors, only two configurations have been found. In the 1990s, an enzyme electrode based on the immobilization of 3 α -HSD on the surface of glassy-carbon and low-temperature isotropic-carbon electrodes by intermolecular cross-linking with bovine serum albumin and glutaraldehyde was described [10]. The linear calibration range extended between 5 and 96 μ M androsterone using a detection potential of +0.6 V. More recently, a configuration making use of

* Corresponding author. Fax: +34 913944329.

E-mail address: yseo@quim.ucm.es (P. Yáñez-Sedeño).

a screen printed Ir/C electrode modified with 3α -HSD was applied to the determination of bile acids, a group of acids produced by the degradation of cholesterol in the liver [11].

The extremely attractive properties of CNTs for the electrochemical detection of H_2O_2 and NADH have led to the development of a great number of enzyme biosensors exhibiting rapid electron transfer at the electrode substrate [12]. Among them, carbon nanotubes paste electrodes (CNTPEs), prepared by mixing CNTs with a binder material, show several practical advantages. First, the resulting electrodes retain the properties of conventional carbon paste electrode (CPE) such as the feasibility to incorporate different substances into the paste matrix, the low background currents achieved, the easy renewal and the composite nature [13,14]. Furthermore, these electrodes combine the ability of carbon nanotubes to promote electron-transfer reactions with the attractive advantages of composite materials. Enzymes or other substances can be physically incorporated into the mixture without the need of covalent bonds, and so, the hybrid composite maintains the properties of CNTs unchanged. On the other hand, the use of room temperature ionic liquids (RTILs) as the binder material for the preparation of CNTPEs improves still more the commented advantages [15]. RTILs are ionic media resulting from the combination of organic cations and various anions, which have wide use as nonaqueous, polar, environmentally friendly solvents. Regarding electrochemistry, RTILs are particularly interesting since they display a wide electrochemical potential window and high ionic conductivity. Moreover, composite materials constituted of MWCNTs and the ionic liquid octylpyridinium hexafluorophosphate (OPPF₆) have shown lower background currents compared to graphite and mineral oil CPEs [16]. Furthermore, these electrodes exhibit good activity toward NADH oxidation as well as a marked electrode stability and antifouling features. These capacities can be profited for the development of enzyme biosensor.

In this work we describe the preparation of MWCNTs/OPPF₆ composite electrodes as platforms for immobilization of the 3α -HSD enzyme in order to construct a 3α -hydrosteroid biosensor for androsterone determination. Fig. 1 shows a scheme of the composition and functioning of the biosensor. As a novelty with respect to other dehydrogenase based configurations, the oxidized form of nicotinamide adenine dinucleotide (NAD^+) was incorporated into the composite matrix. This integration constitutes an efficient strategy for the improved actuation of the co-factor in the

hydrogenase reaction, making possible the fast, sensitive and stable detection of the formed NADH. Very few configurations based on the incorporation of NAD^+ to the electrode matrix have been described in the literature. A lactate biosensor involving the immobilization of both lactate dehydrogenase and NAD^+ on a conducting polymer–MWCNTs composite film electrode was described [17]. Carbon paste electrodes modified with glycerol oxidase and NAD^+ and coated with a poly(*o*-phenylenediamine) film were used for the determination of glycerol in a plant-extract syrup [18]. Glucose dehydrogenase (GDH) biosensors were prepared by covalent attachment of N^1 -carboxymethyl NAD^+ species to polyamino-saccharide chains of chitosan (Chit) and cross-linking with glutaraldehyde (GDI). The Chit– NAD^+ –GDH–GDI–Chit mixture was adsorbed onto the carbon nanotubes and deposited onto glassy carbon electrodes [19]. More recently, a simpler and faster procedure was described for the preparation of a glucose dehydrogenase biosensor based on modification of glassy carbon electrodes with MWCNTs/ NAD^+ composite, where the strong π – π stacking interaction between the adenine moiety in the cofactor and carbon nanotubes provides a high stability [20].

2. Experimental

2.1. Reagents and solutions

3α -Hydroxysteroid dehydrogenase (3α -HSD), from *Pseudomonas testosteroni* H1506 (Sigma) was used. Enzyme solutions were prepared by dissolving the product (50 units of enzyme) in 500 μL of 0.1 M phosphate buffer solution of pH 7.4 (PBS). A stock 1 mM androsterone (3α -hydroxy- 5α -androstan-17-one) (Fluka) solution was prepared by dissolving 7.5 mg of the product in 25 mL of a 1:1 acetonitrile–0.1 M PBS solution. More diluted androsterone solutions were prepared from this one by diluting it with 0.1 M PBS. *n*-Octylpyridinium hexafluorophosphate (OPPF₆) (Io-Li-Tec), oxidized nicotinamide adenine dinucleotide (NAD^+) (Gerbu) and nicotinamide adenine dinucleotide (reduced form) (NADH) (Sigma) were also used. Multi-walled carbon nanotubes (MWCNTs, $\varnothing 30 \pm 15$ nm) with 95% purity were supplied from NanoLab (Brighton, MA). 0.1 M TRIS buffer solution of pH 8.5 was prepared from Tris HCl (Scharlau). Stock 10^{-3} M cortisol (hydrocortisone), 19-nortestosterone (nandrolone), β -estradiol and testosterone solutions were prepared in 1:1 acetonitrile–PBS. More

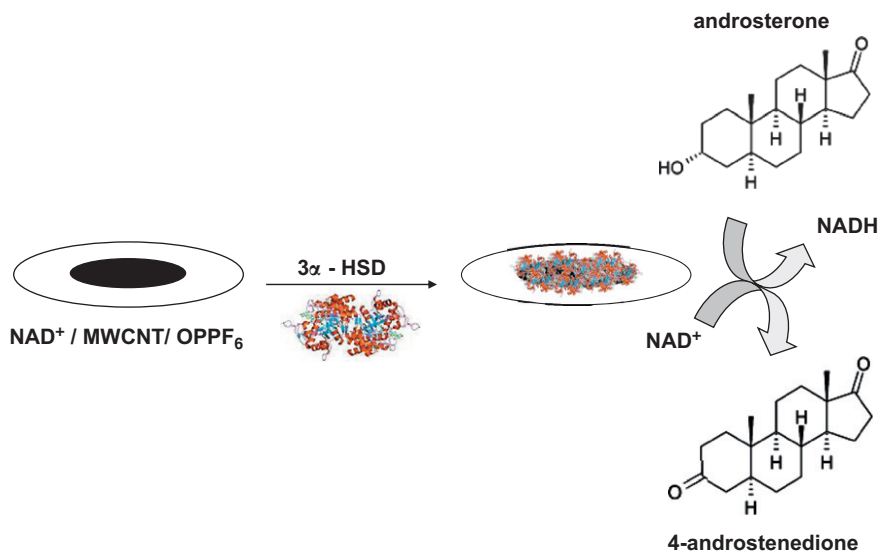


Fig. 1. Scheme displaying the preparation and functioning of the biosensor.

diluted solutions of these compounds were prepared by diluting it with 0.1 M PBS and used for the interference study. Other solvents and chemicals used were of analytical reagent grade and water was obtained from a Millipore Milli-Q purification system.

2.2. Samples

Analyzed samples were lyophilized human serum from Sigma spiked with androsterone at the 1.25 μM (0.36 $\mu\text{g/mL}$) concentration level. Spiked samples were prepared by adding 50 μL of standard 50 μM androsterone solution to 2.0 mL of serum and they were stored at -40°C until analyzed. Control serum samples (non-spiked) were prepared in 0.1 M PBS pH 7.4.

2.3. Apparatus and electrodes

Amperometric measurements were carried out using an amperometric detector from InBea Biosensores SL, Madrid, Spain. Cyclic voltammograms were obtained using a PGSTAT 12 potentiostat from Autolab. The electrochemical software was the general-purpose electrochemical system (GPES) (EcoChemie B.V.). A three electrodes cell (BAS VC-2 10 mL glass electrochemical cell) equipped with a platinum wire counter electrode (BAS MW-1032), a CHI 111 Ag/AgCl/3 M KCl reference electrode, and the MWCNTs/OPPF₆/NAD⁺ electrode, or the 3 α -HSD-enzyme biosensor as the working electrodes, were used. All experiments were performed at room temperature.

2.4. Procedures

2.4.1. Preparation of 3 α -HSD/ MWCNTs/ OPPF₆/ NAD⁺ biosensors

Composite MWCNTs/OPPF₆/NAD⁺ electrodes were fabricated from 30 mg MWCNTs, 70 mg OPPF₆ and 20 mg NAD⁺, which were thoroughly mixed until complete homogenization. Portions of the resulting mixture were packed into Teflon holders (3 mm inner diameter) and pressed tightly. The electrical contact was made through a stainless steel screw. Subsequently, a 10 μL aliquot of 3 α -HSD enzyme solution was deposited onto the electrode surface, and allowed to dry overnight at room temperature in darkness.

2.4.2. Determination of androsterone in serum samples

2.0 mL of the sample prepared and stored as described in Section 2.2 was diluted to 5.0 mL with 0.1 M TRIS buffer solution of pH 8.5. Androsterone was determined by amperometry under continuous stirring at a detection potential of +400 mV, which allowed monitoring the oxidation of the generated NADH, and using the standard additions method implying successive additions of 100 μL from 2.5×10^{-5} M androsterone solution.

3. Results and discussion

3.1. Preparation of the 3 α -HSD/MWCNTs/OPPF₆/NAD⁺ biosensor

Firstly, binary MWCNTs/OPPF₆ composites prepared with different carbon nanotubes-ionic liquid percentages were investigated in order to select the optimum ratio for further work. Cyclic voltammetry from 5 mM Fe(CN)₆^{3-/4-} in 0.1 M KCl solutions was used for such purpose (Fig. 2). As it can be observed, voltammograms exhibited larger currents as the MWCNTs/OPPF₆ ratio increased, as a consequence of the enhanced active surface area and the double-layer capacitance [16]. However, a large increase in the background current together with a higher instability of the composites were observed when the ionic liquid percentage was lower than 70%. Therefore, a 30:70 MWCNTs/OPPF₆ composition was selected to

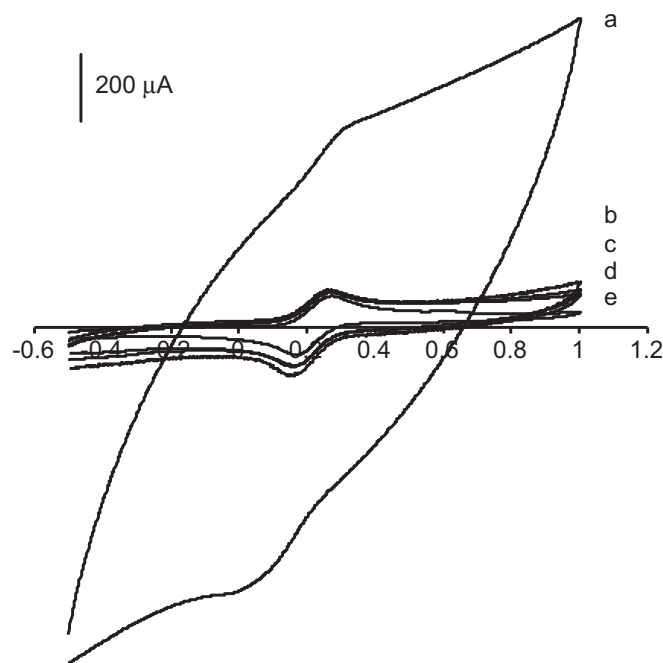


Fig. 2. Cyclic voltammograms of 5 mM Fe(CN)₆^{3-/4-} in 0.1 M KCl recorded at (a) 36/64, (b) 35/65, (c) 30/70, (d) 20/80, and (e) 10/90 MWCNTs/OPPF₆ composite electrodes; $v = 100$ mV/s.

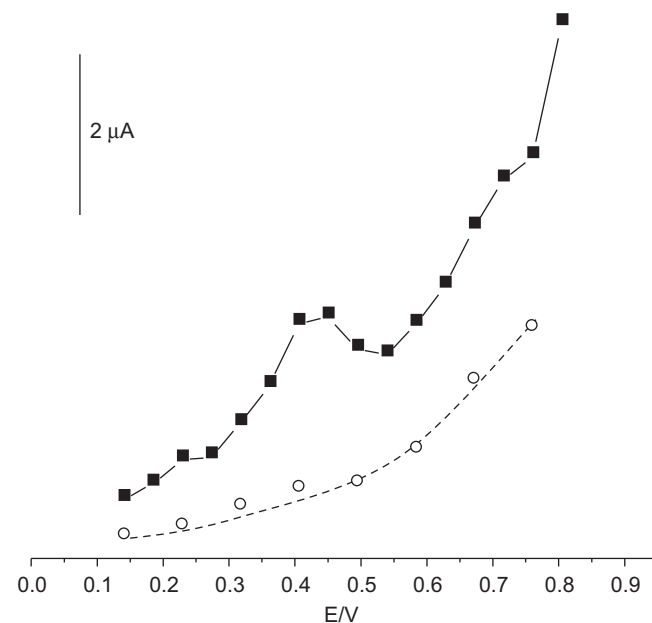


Fig. 3. Effect of the applied potential on the steady-state current measured at a 30/70 MWCNTs/OPPF₆ composite electrode for 5 mM NADH in 0.1 M PBS of pH 7.4. Dotted line, supporting electrolyte.

prepare the electrodic matrices, which exhibited suitable mechanic and conducting properties.

The MWCNTs/OPPF₆ electrode was further used to test the electrochemical behavior of NADH. The results obtained on the influence of the applied potential on the steady-state current measured for a 5 mM NADH solution is displayed in Fig. 3. As it can be observed, significant anodic currents were recorded at the modified electrode for potential values higher than +300 mV. As a compromise between sensitivity and selectivity, a detection potential of +400 mV was selected for further work. At this potential value, a calibration plot was obtained for NADH in the

$2.0\text{--}12.0 \times 10^{-5}$ M concentration range in 0.1 M PBS of pH 7.4 with a slope value of $32.6 \mu\text{A}/\text{mM}$. This value ranks among the highest reported for other composite electrodes using MWCNTs [21,22].

The NAD^+ loading incorporated into the composite electrodes was optimized by testing the amperometric responses of 2.0, 4.0 and 6.0×10^{-6} M androsterone solutions at various $3\alpha\text{-HSD}/\text{MWCNTs}/\text{OPPF}_6/\text{NAD}^+$ biosensors prepared by depositing $10 \mu\text{L}$ of the $3\alpha\text{-HSD}$ enzyme solution onto the electrode surface. The electrodes were prepared with different amounts of NAD^+ in the 0–40% weight range (mg NAD^+ per 100 mg paste). The results obtained (Fig. 4) show as the slope value of the calibration plots constructed for androsterone increased with the NAD^+ loading up to 20% NAD^+ . Higher NAD^+ percentages produced a decrease in the measured currents that can be attributed to the inhibitory effect produced by high cofactor concentrations [23]. The influence of pH on the amperometric response for androsterone was also tested over the 6.0–10.0 range using different 0.1 M phosphate buffer solutions. The slope value of the responses measured for the three androsterone concentrations at the $3\alpha\text{-HSD}/\text{MWCNTs}/\text{OPPF}_6/\text{NAD}^+$ showed a sharp increase between pH 7.0 and 8.5 and a remarkable decrease for higher values (results not shown). This behavior appreciably agrees with previous studies [10]. In order to get the highest sensitivity, pH 8.5 was selected for further work. Moreover, 0.1 M TRIS buffer solution was chosen to regulate that pH value. Under these conditions, the possible leakage of NAD^+ from the modified electrode surface to the electrolyte solution was evaluated. A calibration plot for NAD^+ was constructed by measuring absorbance at 261 nm in the TRIS buffer of pH 8.5 solution. After continuous recording of five cyclic voltammograms with a single MWCNTs/OPPF₆/NAD⁺ modified electrode, interpolation of the absorbance measured in the electrolyte solution into the calibration plot showed that approximately 4% of the NAD^+ confined in the carbon nanotubes paste was leaked from the electrode. Therefore not a very significant leakage of this molecule from the electrode surface was produced under the selected experimental conditions.

The enzyme loading onto the electrode surface was also optimized. The slope values of the calibration graphs obtained for androsterone in the $0\text{--}8.0 \times 10^{-6}$ M concentration range were measured with different biosensors constructed with $3\alpha\text{-HSD}$ loadings between 0.3 and 1.0 enzyme units. The results (not shown) showed that the highest slope value was achieved when 1.0 unit of $3\alpha\text{-hydroxysteroid dehydrogenase}$ was deposited onto the MWCNTs/OPPF₆/NAD⁺ electrode surface. The use of higher enzyme loadings or the incorporation of enzyme into the electrode composite matrix were not considered in order to avoid the increasing biosensor cost.

It is important to remark that cyclic voltammograms from androsterone recorded with a MWCNTs/OPPF₆/NAD⁺ modified electrode did not show any observable voltammetric response. Moreover, amperometric measurements for androsterone carried out with MWCNTs/OPPF₆/NAD⁺ and $3\alpha\text{-HSD}/\text{MWCNTs}/\text{OPPF}_6/\text{NAD}^+$ electrodes revealed that only suitable amperometric responses were obtained with the enzyme electrode.

3.2. Calibration graph and analytical characteristics

Under the optimized working conditions described above, a calibration plot for androsterone was constructed with the $3\alpha\text{-HSD}/\text{MWCNTs}/\text{OPPF}_6/\text{NAD}^+$ biosensor exhibiting a linear range ($r=0.998$) in the $0.5\text{--}10 \mu\text{M}$ concentration range with a slope of $70.3 \mu\text{A}/\text{mM}$. This value is more than two hundred times higher than that obtained with a glassy carbon electrode modified with $3\alpha\text{-HSD}$ and glutaraldehyde at a detection potential of +600 mV [10], in spite of the lower operating potential value used with the $3\alpha\text{-HSD}/\text{MWCNTs}/\text{OPPF}_6/\text{NAD}^+$ biosensor. The detection limit (LOD), $0.15 \mu\text{M}$, was calculated according to the $3sb/m$ criterion, where m is the slope of the linear portion in the calibration graph, and sb was estimated as the standard deviation ($n=10$) of the amperometric signals measured for different androsterone solutions at the lowest concentration level of the calibration graph ($0.5 \mu\text{M}$). Successive amperometric measurements of five independent $6.0 \mu\text{M}$ androsterone solutions carried out with the same biosensor provided a relative standard deviation (RSD) value of 1.8%. Furthermore, the reproducibility of the responses obtained with five different $3\alpha\text{-HSD}/\text{MWCNTs}/\text{OPPF}_6/\text{NAD}^+$ enzyme sensors was also evaluated, yielding a RSD value of 3.5%. These results show fairly well the good repeatability and reproducibility achieved with the developed biosensor.

The enzyme reaction at the $3\alpha\text{-HSD}/\text{MWCNTs}/\text{OPPF}_6/\text{NAD}^+$ composite biosensor fitted well into a Michaelis–Menten kinetics, as it was demonstrated by calculation of the “ x ” parameter (0.99 ± 0.08) from the corresponding Hill’s plot ($[\log(i_{\text{max}}/i) - 1]$ vs. the log of androsterone concentration). Therefore, the apparent Michaelis–Menten constant ($K_{\text{app}}^{\text{M}}$), $36.0 \mu\text{M}$, was calculated from the corresponding Lineweaver–Burk plot. The $K_{\text{app}}^{\text{M}}$ value obtained was much lower than that reported in the literature by immobilization of $3\alpha\text{-HSD}$ onto a glassy carbon electrode, $189 \mu\text{M}$ [10], and is similar to that obtained for the enzyme in a solution of pH 9 using UV–visible spectrophotometry, $30 \pm 3 \mu\text{M}$ [9]. The low $K_{\text{app}}^{\text{M}}$ value obtained reflects a high affinity for the substrate when the enzyme is immobilized in the carbon nanotubes/liquid ionic microenvironment, conversely to

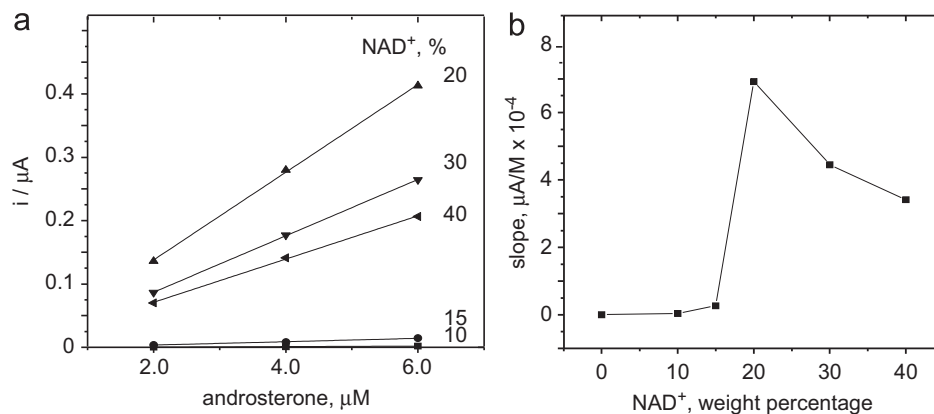


Fig. 4. Influence of the NAD^+ loading in the $3\alpha\text{-HSD}/\text{MWCNTs}/\text{OPPF}_6/\text{NAD}^+$ biosensor on the amperometric responses measured for $2.0\text{--}6.0 \mu\text{M}$ androsterone solutions in 0.1 M pH 8.5 Tris buffer. $E_{\text{app}}=0.4$ V.

that occurred upon immobilization on a carbon disk electrode where enzyme deactivation proceeds rapidly.

The lifetime of a single 3α -HSD/MWCNTs/OPPF₆/NAD⁺ biosensor was checked by performing daily three calibration plots for androsterone in the 2.0–6.0 concentration range. The mean value of the slopes of these calibrations did not show significant differences (within $\pm 3 \times$ standard deviation of the mean value measured the first day) for at least 6 days, without applying any regeneration procedure to the bioelectrode surface. Therefore, no significant loss the 3α -HSD enzyme bioactivity or re-dissolution into the electrolyte solution was observed for the above mentioned period of time.

3.3. Interferences study

Various steroid hormones structurally related with androsterone: testosterone, β -estradiol, 19-nortestosterone (nandrolone) and cortisol (Fig. 5), as well as uric and ascorbic acids, were tested as potential interfering compounds. Amperometric currents were measured in 0.1 M Tris buffer pH 8.5 with 3α -HSD/MWCNTs/OPPF₆/NAD⁺ biosensors for different interfering/androsterone concentration ratios. Among the hormones tested, only β -estradiol produced a significant interference under the experimental conditions used, giving a relative error higher than 5% from an interferent/androsterone ratio of 0.05. This result agreed with the reported slight 17- β -estradiol-dehydrogenase activity from pure 3α -HSD [24]. However, this concentration is various orders of magnitude higher than the physiological level of β -estradiol in serum samples. Furthermore, cortisol and 19-nortestosterone, at concentrations ten times higher than the respective physiological levels, and testosterone, at a concentration 1000 times higher than its physiological level did not produce any noticeable interfere on the biosensor response to androsterone.

On the other hand, uric and ascorbic acids were electrochemically oxidized at the potential value used for androsterone detection, and, therefore, interference from both compounds was observed from 1.0×10^{-5} to 1.0×10^{-6} M, with relative errors of 3.8% and 3.7%, respectively. The extent of these interferences could be largely minimized by coating the electrode surface with a Nafion film, though at the expense of a noticeable decrease of the calibration slope value. Nevertheless, as it will be

demonstrated below, the determination of androsterone in human sera with the 3α -HSD/MWCNTs/OPPF₆/NAD⁺ biosensor implied a procedure that allowed a negligible interference to be observed from these endogenous compounds.

3.4. Determination of androsterone in serum samples

The usefulness of the developed enzyme biosensor was evaluated by means of the analysis of human serum samples spiked with androsterone at a 1.25 μ M (0.36 μ g/mL) concentration level. As it is described in Section 2.4.2, 2.0 mL of reconstituted serum was diluted to 5 mL with 0.1 M Tris buffer solution of pH 8.5, and the determination of androsterone was carried out by applying the standard additions method involving 100 μ L additions from a 2.5×10^{-5} M androsterone standard solution. Serum samples with no androsterone did not provide significant amperometric current values at +400 mV, thus demonstrating the absence of interference from endogenous compounds. Mean recoveries obtained ($n=5$) ranged between 97.2% and 103% with a relative standard deviation of 2.7%, which demonstrated fairly well the usefulness of the biosensor for the rapid analysis of androsterone in human serum samples. These results demonstrated also the absence of significant interference from other compounds present in the human serum samples, thus confirming the validity of the methodological approach.

4. Conclusions

A novel enzyme electrode platform, involving a mixture of MWCNTs, an ionic liquid, the incorporation of NAD⁺ cofactor into the electrode matrix and immobilization of 3α -HSD, has been developed for the determination of the hormone androsterone. The analytical characteristics of the constructed biosensor fulfill the requirements for the analysis of the hormone in human serum samples. Moreover, the versatility of the approach allows direct extrapolation of the strategy to prepare dehydrogenase-based enzyme biosensors in a straightforward manner.

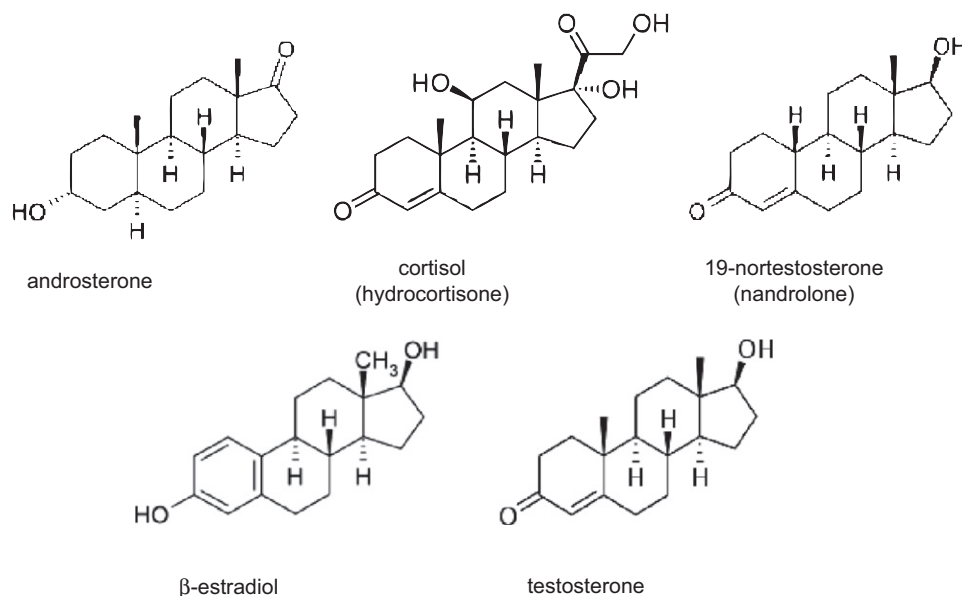


Fig. 5. Structures of androsterone and related compounds.

Acknowledgments

Financial support from the Ministerio de Ciencia e Innovación (Projects DPS2008-07005-C02-01 and CTQ2009-12650), and Comunidad de Madrid S2009/PPQ-1642 (AVANSENS), is gratefully acknowledged.

References

- [1] C. Saudan, N. Baume, P. Mangin, M. Saugy, J. Chromatogr. B 810 (2004) 157–164.
- [2] B.O. Dubrovsky, Prog. Neuro-Psychopharmacol. 29 (2005) 169–192.
- [3] P. Gärtner, C. Novak, C. Einzinger, W. Felzmann, M. Knollmüller, G. Gmeiner, W. Schänzer, Steroids 68 (2003) 85–96.
- [4] T. Trinh, N.B. Harden, H.M. Coleman, S.J. Khan, J. Chromatogr. A 1218 (2011) 1668–1676.
- [5] P. Van Eenoo, W. Van Gansbeke, N. De Brabanter, K. Deventer, F.T. Delbeke, J. Chromatogr. A 1218 (2011) 3306–3316.
- [6] R. Aguilera, T.E. Chapman, H. Pereira, G.C. Oliveira, R.P. Illanes, T.F. Fernandes, D.A. Azevedo, F.A. Neto, J. Steroid Biochem. 115 (2009) 107–114.
- [7] A. Ishizaki, H. Kataoka, J. Pharmacol. Biol. Anal 52 (2010) 727–733.
- [8] O.J. Pozo, K. Deventer, P.V. Eenoo, F.T. Delbeke, Anal. Chem. 80 (2008) 1709–1720.
- [9] T.M. Penning, I. Mukharji, S. Barrows, P. Talalay, Biochem. J. 222 (1984) 601–611.
- [10] M. Teodorczyk, W.C. Purdy, Talanta 37 (1990) 795–800.
- [11] B. Bartling, L. Li, C.-C. Liu, Analyst 134 (2009) 973–979.
- [12] L. Agüí, P. Yáñez-Sedeño, J.M. Pingarrón, Anal. Chim. Acta 622 (2008) 11–47.
- [13] J. Wang, M. Musameh, Anal. Chem. 75 (2003) 2075.
- [14] M.D. Rubianes, G.A. Rivas, Electrochem. Commun. 5 (2003) 689–694.
- [15] M.J.A. Shiddiky, A.A.J. Torriero, Biosens. Bioelectron. 26 (2011) 1775–1787.
- [16] R.T. Kachooangi, M.M. Musameh, I. Abu-Yousef, J.M. Yousef, S.M. Kanan, L. Xiao, S.G. Davies, A. Russell, R.G. Compton, Anal. Chem. 81 (2009) 435–442.
- [17] M.M. Rahman, M.J.A. Shiddiky, Md.A. Rahman, Y.-B. Shim, Anal. Biochem. 384 (2009) 159–165.
- [18] M.I. Álvarez-González, S.B. Saidman, M.J. Lobo-Castañón, A. Miranda-Ordieres, P. Tuñón-Blanco, Anal. Chem. 72 (2000) 520–527.
- [19] M. Zhang, C. Mullens, W. Groski, Anal. Chem. 79 (2007) 2446–2450.
- [20] H. Zhou, Z. Zhang, P. Yu, L. Su, T. Ohsaka, L. Mao, Langmuir 26 (2010) 6028–6032.
- [21] M. Pumera, A. Merkoci, S. Alegret, Sensors Actuators B Chem. 113 (2006) 617.
- [22] J. Manso, M.L. Mena, P. Yáñez-Sedeño, J.M. Pingarrón, Electrochim. Acta 53 (2008) 4007–4012.
- [23] S. Chakraborty, C. Retna Raj, Electrochem. Commun. 9 (2007) 1323.
- [24] E. Battais, B. Terouanne, J.C. Nicolas, B. Descomps, A. Crastes de Paulet, Biochimie 59 (1977) 909–917.