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Determination of progesterone (P4) from bovine serum samples using a microfluidic immunosensor system

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ABSTRACT

Progesterone (P4) is a steroidal hormone with a vital role in the maintenance of human and animal health. This paper describes the development of an immunosensor coupled to glassy carbon (GC) electrode and integrated to a microfluidic system to quantify P4 from bovine serum samples in a fast and sensitive way. The serum samples spiked with a given P4 concentration and a given P4 concentration bound to horseradish peroxide (HPR) were simultaneously added and, therefore, they competed immunologically with sheep monoclonal anti-P4 antibodies that were immobilized at a rotating disk. HRP in the presence of hydrogen peroxide (H_2O_2) catalyzes the chatecol (H_2Q) oxidation to benzoquinone (Q). Its reverse electrochemical reduction to H_2Q can be detected at a GC electrode surface at −0.15 V by chronoamperometric measurements. These current responses are proportional to the enzyme activity and inversely proportional to the P4 amount present in bovine serum samples. This P4 immunosensor showed a linear working range from 0.5 to 12.5 ng mL^{-1}. The detection (DL) and quantification (QL) limits were 0.2 and 0.5 ng mL^{-1}, respectively. The electrochemical immunosensor had a higher sensitivity than the ELISA method using conventional spectrophotometric detections. However, both methods allowed us to obtain similar detection limits. The immunosensor allowed us to make up to 100 determinations on different samples without any previous pre-treatment. This behavior proved to be suitable to detect P4 in routine veterinary, clinical, biological, physiological, and analytical assays.

1. Introduction

Nowadays, the use of electrochemical immunosensors as efficient analytical tools in the fields of clinical diagnosis and environmental monitoring is well established [1,2]. These immunosensors have excellent analytical applications, such as higher sensitivity and reproducibility. Their construction and use is very simple. These important advantages allow the electrochemical immunosensors be able to perform immunoreagent immobilizations, be more efficient in transduction events as well as work in a small scale.

Progesterone (P4) (pregn-4-ene-3,20-dione) is an α,β unsaturated ketone, a steroid hormone with a vital role in the maintenance of human and animal health. Its imbalance can cause malformations in the reproductive system as well as infertility problems [3,4]. P4 is secreted by the uterus to prepare it for pregnancy and maintain the pregnancy after conception secretes P4. Therefore, P4 is adequate to diagnose an early pregnancy [5]. P4 levels should be at sufficiently high values in the organism to assure the embryo development [6]. Thus, monitoring of P4 levels is important to reproductive system. A periodic control of P4 level is used to determine the next most fertile ovulation time and to program the animal's service or the artificial insemination, which would help to improve the percentages of pregnancy [7,8].

The most common methods to quantify P4 in animal serum are those based on enzyme-linked immunosorbet assay (ELISA) and radio immuno assay (RIA). These methods are applied using commercial kits [8,9,10]. However, immobilized antibodies have to be discarded immediately after the first use in these assays. HPLC is another technique used to quantify P4, where ethanol–water solvent binary mixtures are employed as the mobile phase and the column should be thermostatized to 40 °C [11]. Hart et al. [12] have designed a carbon paste amperometric biosensor to detect P4 indirectly in milk through determinations of products involved in the enzymatic-substrate reaction. Another biosensor has also been designed by Claycomb et al. [13,14], which uses the same enzymatic immunoassay method to detect P4 in milk. Recently, an amperometric immunosensor was developed using a colloidal gold–graphite–Teflon–tyrosinase composite to determine P4 in milk [15]. Nevertheless, the implementation of sensitive, fast and

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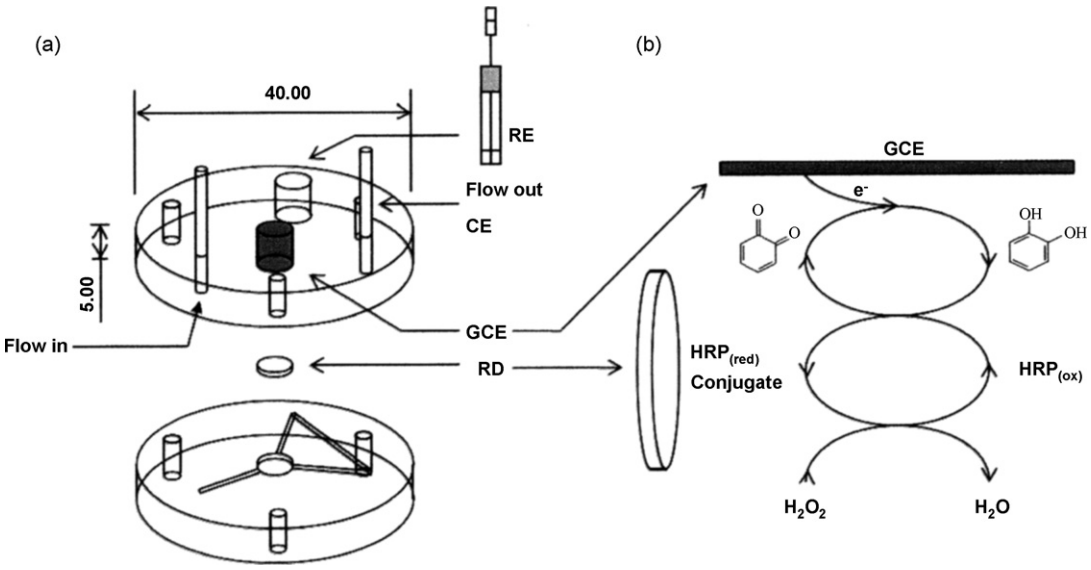


Fig. 1. (a) Schematic representation of the microfluidic sensor cell. (RE) Reference electrode, (CE) counter electrode, (RD) rotating disk. Units of length are given in millimeters. Flow channels i.d.: 0.100 mm. (b) Schematic representation of the reduction wave of the enzymatic process among chatecol (H_2Q), benzoquinone (Q), hydrogen peroxide (H_2O_2), and P4 conjugated with HRP.

low cost methodologies to quantify P4 in bovine serum samples is still a challenge to carry out further studies.

Heterogeneous enzymatic immunoassays, coupled with amperometric detection flow injection (FI) system, represent a powerful analytical tool to determinate low levels of different analytes, such as hormones, antibodies, drugs, tumor markers, and viruses [16]. The amperometric detection offers a good sensitivity combined with a simple and low-cost instrumentation [17,18]. One approach used is to employ a labeled enzyme which generates an electrochemically active product [19–22].

In this paper, we study the development of an immunosensor coupled to a glassy carbon (GC) electrode integrated to a microfluidic system to quantify P4 in bovine serum samples previously spiked with a known P4 concentration. A fast and sensitive method based on the use of a monoclonal antibody (mAb), which was immobilized on a rotating disk. Bovine serum samples containing P4 were added to the reactor with a known concentration of enzyme-labeled antigen (i.e., the "conjugated"). Therefore, labeled and unlabeled antigens compete with antibody binding sites bound at the rotating disk. The enzyme used to label the antigen was horseradish peroxidase (HRP) and chatecol (H_2Q) was the enzymatic redox mediator. HRP in the presence of hydrogen peroxide (H_2O_2) catalyzes the oxidation of H_2Q to benzoquinone (Q). Its reverse electrochemical reduction to chatecol can be detected on the GC electrode surface at −0.15 V through chronoamperometric measurements. These amperometric responses are proportional to the activity of the enzyme and inversely proportional to the amount of P4 in bovine serum samples.

2. Experimental

2.1. Chemicals

All reagents used were of analytical reagent grade. P4, progesterone–3-carboxymethyloxime (P4–3-CMO), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC), sheep monoclonal antibody (mAbP4) and horseradish peroxidase (HRP) were obtained from Sigma Chemical Company and *N*-hydroxysuccinimide (NHS) from Fluka. Anhydrus dimethylformamide (DMF), Glutaraldehyde (25% aqueous solution) and pH 7.00 phosphate buffer solutions (PBS) as well as H_2O_2 were purchased from Aldrich and Merck (Darmstadt, Germany), respectively. H_2Q was obtained from BDH Chemicals Ltd. (Poole, England). All reagents were used as received. 3-Aminopropyl-modified controlled pore glass, 1400 Å mean pore diameter and 24 $m^2\,mg^{-1}$ surface areas, was obtained from Electro Nucleonics (Fairfield, NJ, USA) and contained 48.2 $\mu mol\,g^{-1}$ of amino groups. The School of Agriculture and Veterinary from Universidad Nacional of Río Cuarto gently supplied bovine serum samples. The ELISA test kits for P4 quantitative determination were obtained from Abbott Axsym System®, Reagent Pack, Abbott Japan Co., Ltd. The manufacturer's instructions were taken into account to perform these assays. Aqueous solutions were prepared using purified water from a Milli-Q system.

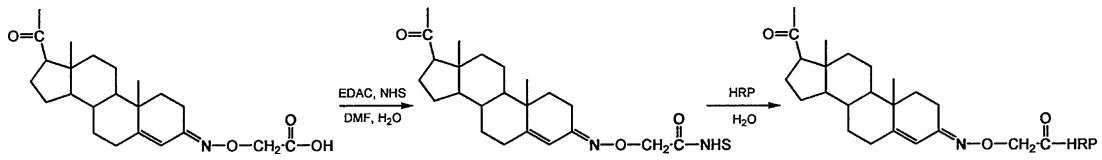
2.2. Apparatus

The main body of the cell was made of Plexiglas. Both the design of the flow through chamber containing the microfluidic immunosensor and its detection system are shown in Fig. 1a.

The volume of the cell was 5 μL. The GC electrode was on the top of the sensor. The rotating reactor was a disk of Teflon (3 mm diameter) where a miniature magnetic stirring bar was incorporated. 0.3 mg of controlled-pore glass is stuck on its surface. The reactor rotation was performed by a magnetic stirrer (Metrohm E649 from Metrohm AG, Herisau, Switzerland) and controlled with a variable transformer of an output between 0 and 250 V and maximum amperage of 7.5 A (Waritrans, Argentina). Chronoamperometry and cyclic voltammetry measurements were performed by using a BAS LC4C and a BAS 100 B (electrochemical analyzer from Bioanalytical Systems, West Lafayette, IN, USA), respectively.

The potential applied to the GC electrode for performing amperometric measurements was −0.15 V *vs* Ag/AgCl, 3.0 $mol\,L^{-1}$ NaCl reference electrode (BAS RE-6). A Pt wire was the counter electrode. A catalytic current was established at this potential. The inner diameter (i.d.) of flow channels was 0.100 mm and the length of flow channels was 10 mm. Pumps (Baby Bee Syringe Pump, Bioanalytical System, West Lafayette, IN) were used for pumping, introducing samples and stopping the flow.

The pH measurements were carried out with an Orion Expandable Ion Analyzer (model EA 940, Orion Research, Cambridge, MA, USA) equipped with a glass combination electrode (Orion



Scheme 1. Synthesis of P4–3-CMO with HRP to form the labeled P4.

Research). Absorbance measurements were performed by Bio-Rad Benchmark microplate readers (Japan) and Hewlett-Packard spectrophotometer, Model 8452A, equipped with a temperature controller.

2.3. Preparation of P4–HRP conjugate

P4 was bound to HRP by following a synthesis of activated ester to generate the P4 labeled. Thus, 7.9 mg of P4–3-CMO were dissolved in 600 μL of DMF containing 400 μL of distilled water and 18 mg of NHS. Then, 33 mg of EDAC were added. Reaction mixture was stirred to 4 °C in the darkness for 5 h to activate the carboxyl group of the steroid. HRP (1 mg) was dissolved in 1 mL of distilled water. Steroid activated solution was added to HRP aqueous solution and then stored and shaken overnight at 4 °C. The solution was then dialyzed in water for 5 days by using a cellulose dialysis membrane (cut-off molecular weight 12 kDa). Finally, it was stored at 4 °C (see Scheme 1).

Concentrations of synthesized P4–HRP conjugated (P4–HRP) were determined spectrophotometrically by measuring absorbance changes at 252 and 402 nm in 0.01 mol L^{-1} PBS (pH 7.0), considering that P4–3-CMO and HRP show absorption maximum at those wavelength, respectively. The synthesized conjugated P4–HRP had 8 mol of P4 per HRP mole as it was determined spectrophotometrically. This ratio showed that there was unbound P4 in the solution. The true synthesized P4–HRP conjugated concentration was unknown. Therefore, different known dilutions were prepared for convenience.

2.4. Progesterone immunoassay

The ELISA test kits with standard series covering the relevant range (0–25 ng mL^{-1}) were supplied by the company, as mentioned before [23]. The manufacturer's protocol was followed to perform standard calibration curves from spectrophotometric measurements. Therefore, P4 concentrations were calculated measuring absorbance changes at 405 nm.

2.5. mAbP4 immobilization

The rotating disk reactor (bottom part, Fig. 1a) was prepared by immobilizing mAbP4 on a controlled pore glass modified by 3-aminopropyl (APCPG), which was smoothly spread on one side of a double-coated tape attached to the disk surface. Then, it was allowed to react with a 5% (w/w) glutaraldehyde aqueous solution of pH 10.0 (0.20 mol L^{-1} carbonate) for 2 h at room temperature. After different washing steps with both purified water and 0.01 mol L^{-1} PBS (pH 7.0), 30 μL of mAbP4 different dilutions were allowed to react with aldehyde residual groups overnight at 5 °C. Then, 30 μL of 0.1 mol L^{-1} glycine solution was added to block the remaining reactive groups for 2 h at room temperature after being thoroughly rinsed with water. Then, these groups were reduced with 30 μL of 0.5% $NaBH_4$ solution for 2 h at room temperature. Finally, the immobilized antibody was washed with 0.01 mol L^{-1} PBS (pH 7.0) and stored in the same buffer at 4 °C between measurements. Immobilized antibody preparations remained stable for at least 1 month.

2.6. Amperometric analysis of P4 in serum bovine samples

The amperometric measurements were applied for P4 determination through a competitive assay in different bovine serum samples, which had been previously spiked with a known P4 concentration. The experimental procedure was performed at a constant rotation speed of 180 rpm and at a flow rate of 50 μL min^{-1}. Unspecific bindings were blocked through a treatment at 37 °C with 3% low-fat milk in a 0.01 mol L^{-1} (pH 7.0) under stopped flow conditions, during 10 min. After that, the immunoreactor was washed with 0.01 mol L^{-1} PBS (pH 7.0) at a flow rate of 50 μL min^{-1} and serum samples and P4–HRP conjugated were then injected into the 0.01 mol L^{-1} PBS (pH 7.0) carrier stream and incubated at 37 °C under stopped flow conditions for 15 min (competitive assay). Then, the immunoreactor was washed by using a 0.01 mol L^{-1} PBS (pH 7.0) at a flow rate of 50 μL min^{-1} in order to eliminate any traces of unbound P4 and P4–HRP. Finally, a substrate solution (10 μL 0.1 mol L^{-1} phosphate–citrate buffer, pH 5.0 + 1×10^{-3} mol L^{-1} H_2O_2 + 1×10^{-3} mol L^{-1} H_2Q) was injected and the enzymatic reaction product was detected through amperometric FI measurements for 2 min under stopped flow conditions.

For the next analysis, the immunoreactor was reconditioned by desorption. First, buffer injections (0.1 mol L^{-1} glycine–HCl, pH 2.0) were applied for 2 min and then, the immunoreactor was washed with 0.01 mol L^{-1} PBS (pH 7.0). The desorption efficiency was checked by the absence of the reduction current of the enzymatic reaction product at GC electrode at −0.15 V after the addition of H_2O_2 and H_2Q, which is a consequence of the absence of P4–HRP.

A standard amperometric curve was generated by employing concentrations which covered the clinically relevant range supplied with ELISA test kits (0–20 ng mL^{-1}). The following protocol was used: amperometric measurements were performed at GC electrode at a potential of −0.15 V at room temperature in 0.1 M phosphate–citrate buffer (pH 5.0) solutions containing 1×10^{-3} mol L^{-1} H_2O_2 + 1×10^{-3} mol L^{-1} H_2Q. The corresponding cathodic peak currents were recorded in the potentiostat meter. The immunoreactor was stored in 0.01 mol L^{-1} PBS (pH 7.0) at 4 °C when it was not used.

3. Results and discussion

3.1. Study of the enzymatic process

Reactions catalyzed by enzymes have been largely used for analytical purposes in determinations of different substrates, inhibitors, etc. Gorton [24] has explained the HRP catalytic mechanism. Therefore, it is well known that in the presence of H_2O_2 the enzyme catalyzes the oxidation of H_2Q to Q [25], which at a potential of −0.15 V can be electrochemically reduced to H_2Q, providing a reduction current peak which can be related to substrate concentration [26]. Fig. 1b shows a schematic representation of this process.

3.2. Optimization of the experimental variables involved on antigen–antibody reaction

mAbP4 concentration is not provided by the Sigma commercial reactive. The synthesized P4–HRP concentration is also unknown,

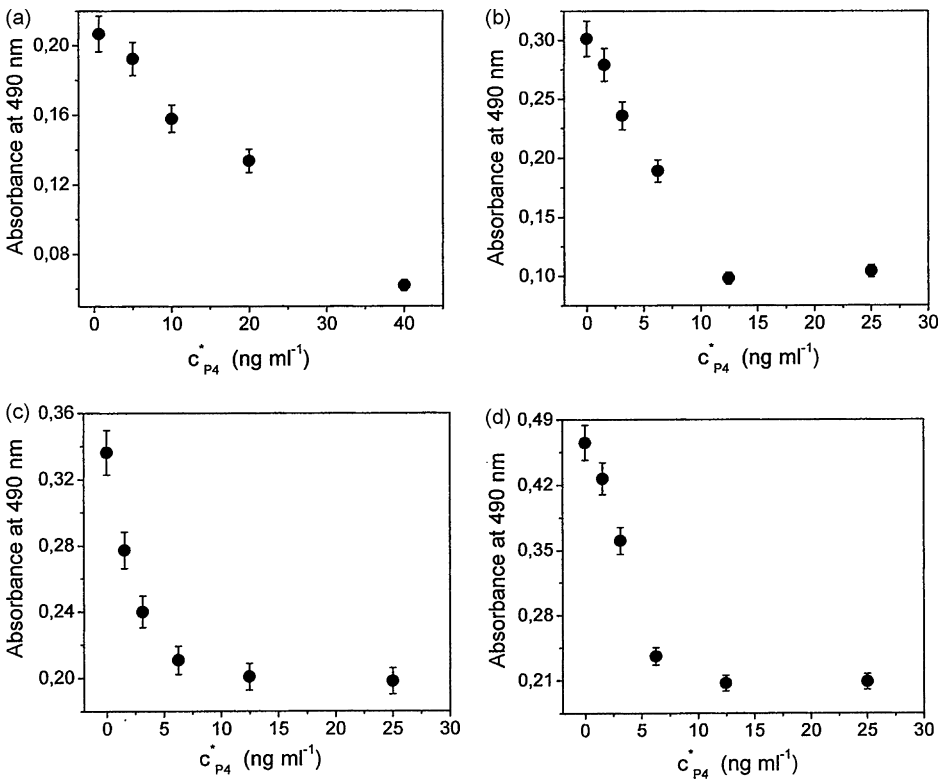


Fig. 2. Optimization of P4–HRP. ELISA assay for different P4–HRP dilution factors. (a) 1/190; (b) 1/100; (c) 1/75 and (d) 1/55. Dilution factor of mAbP4 = 1/50.

so different dilutions were prepared for both. In this study, optimal dilutions for mAb and P4–HRP were studied by using an ELISA test kit. Optimal responses were proved from 1/50 to 1/4000 and 1/10 to 1/1200 dilution ranges for mAb and P4–HRP, respectively.

Curves for each mAbP4 dilution were obtained at different P4–HRP dilutions. It was experimentally observed that the smaller the mAbP4 dilution factor was, the higher the absorbance value was (results not shown). The chosen value for the dilution factor was 1/50 in order to achieve the best sensitivity.

In addition, at a mAbP4 constant dilution factor of 1/50, an increase of P4–HRP concentrations produced also an increase in absorbance values (results not shown). P4–HRP dilution factors smaller than 1/10 produce the saturation of mAbP4 immobilized, since constant absorbance signals were observed when the P4–HRP amount was greater [27]. Different competitive assays between P4–HRP and P4 were carried out in order to know the best dilution factor. Competitive assays for different P4–HRP factor dilutions are shown in Fig. 2.

Absorbance values increased as P4–HRP concentration was increased (lower dilution) but the linear range of P4 concentration decreased due to saturation of mAbP4 adsorbing sites to high P4 concentrations.

The highest linear range with very good responses was obtained by using a 1/100 P4–HRP dilution factor, which coincides with veterinary relevant range (0–15 ng mL^{-1}) [7].

3.3. Effect of reactor rotation and continuous flow/stopped flow operation

The implementation of continuous flow/stopped flow programming was followed by the location of an independent two-phase reactor with two different surfaces, which are facing each other, one is the sensing element itself (GC electrode) and the other is the one where the immune reaction takes place (Teflon disk) (Fig. 1). This design allows for: (I) the use of relatively low immunoreaction loading conditions, (II) an instantaneous operation under high initial rate conditions, (III) the easy detection of accumulated products, and (IV) the reduction of apparent Michaelis–Menten constant K_M'. It is possible to get a complete reagent homogenization because the cell works as a mixing chamber facilitating the arrival of immunoreactants at the specific mAbP4 and enzymatic substrate at active sites as well as the release of products from the same sites [28]. This procedure allows us to obtain higher current values. The main advantages of this system are its simplicity and the convenience to determine P4 in serum samples.

The rotation velocity effect was studied in a range from 60 to 250 rpm under stopped and continuous flow conditions. A linear relationship between the electrical signal and rotation velocity in the range from 60 to 180 rpm under stopped flow conditions was found, showing a response maximum rate for rotation velocities higher than 180 rpm (Fig. 3a). The electrical signal decayed when the reactor operated under continuous flow conditions (Fig. 3b), probably because the reaction product (benzoquinone) escaped from the reactor chamber. When the reactor rotation in the cell stopped, the current response was lower because controlled diffusion reactions were too slow for being detected at the time scale of amperometric measurements, operating under stopped or continuous flow conditions (Fig. 3c and d, respectively). Thus, a rotation velocity of 180 rpm was chosen to be administered to the reactor located at the bottom of the cell under stopped flow conditions (with mAbP4 immobilized and the already formed immunocomplex between P4 of bovine serum samples + the P4–HRP). The sample volume was studied in the range 2–20 μL. Sensitivity was almost tripled in the range between 2 and 10 μL. Insignificant differences were obtained for greater sample size. A sample size of 10 μL had to be injecting in order to minimize convective and dispersive effects that characterize the FI system characteristic as well as to obtain a complete filling of the chamber.

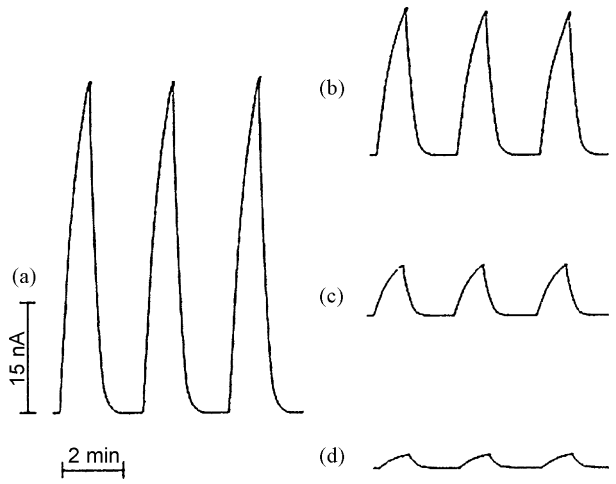


Fig. 3. Effect of reactor's rotation under continuous and stopped flow conditions. (a) Stopped flow with rotation (180 rpm). (b) Continuous flow with rotation (180 rpm). (c) Stopped flow without rotation. (d) Continuous flow without rotation. c^*_{P4} = 3 ng mL^{-1}. Flow rate = 50 μL min^{-1}, cell volume = 5 μL. 0.1 mol L^{-1} phosphate–citrate buffer, pH 5.0 solution containing 1.0×10^{-3} mol L^{-1} H_2O_2 and 1.0×10^{-3} mol L^{-1} H_2Q. The flow was stopped for 2 min during measurement.

3.4. Optimal conditions for the determination of the enzymatic products

The rates of the enzymatic reaction under stopped-flow conditions have been previously studied [28]. These responses showed a maximum value at pH 5.0 in 0.1 mol L^{-1} phosphate–citrate buffer. As it is well known, the optimal temperature of immunoreactions is about 37 °C. Therefore, in this study all experimental measurements were performed at this temperature. The effect of varying H_2O_2 concentration from 7.0×10^{-4} to 5.0×10^{-3} mol L^{-1} at a given H_2Q concentration (1.0×10^{-3} mol L^{-1}) as well as the effect of varying H_2Q concentration from 1.0×10^{-4} to 3.6×10^{-2} mol L^{-1} at a given H_2O_2 concentration (1.0×10^{-3} mol L^{-1}) was evaluated on bioreactor responses. The optimal H_2O_2 and H_2Q concentrations found were 1.0×10^{-3} mol L^{-1} and 1.0×10^{-3} mol L^{-1}, respectively. Those concentrations were, then, used for all experiments.

3.5. Detection and competitive immunoassay on the immunoreactor

Under the previously described selected conditions, amperometric measurements obtained for the reduction of Q in 0.1 mol L^{-1} phosphate–citrate buffer (pH 5.0) were proportional to enzyme conjugated (P4–HRP) activity and, consequently, indirectly proportional to the amount of P4 in serum bovine samples bound to mAbP4 immobilized on the rotating disk. A linear calibration curve for detection of P4 in PBS 0.01 mol L^{-1} (pH 7.0) was obtained. For convenience, sampling times of 2 min at different P4 concentrations were used, considering that longer sampling times than 2 min produced a loss of linear relationship between current responses and P4 concentrations. A good linear calibration curve was found between 0.5 and 12.5 ng mL^{-1} (Fig. 4). For greater concentrations than 12.5 ng mL^{-1}, the saturation of the immobilized mAbP4 took place. The linear regression equation was:

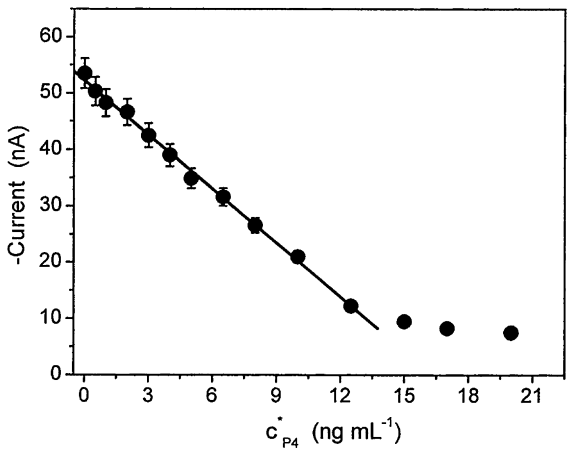


Fig. 4. Calibration curve for P4 determination by amperometric FI immunoassay. Amperometric measurements were performed at −0.15 V *vs* Ag at 37 °C in a 0.1 mol L^{-1} phosphate–citrate buffer (pH 5.0) solution containing 1.0×10^{-3} mol L^{-1} of H_2O_2 and 1.0×10^{-3} mol L^{-1} H_2Q. Current sampling time: 2 min.

$$\text{Current [nA]} = (52.2 \pm 0.9)\,[\text{nA}] - (3.0 \pm 0.1)\,[\text{nA ng}^{-1}\text{ mL}]c^*_{P4}\,[\text{ng mL}^{-1}]$$

being the correlation coefficient, $r = 0.998$. Each experimental point shown in Fig. 4 is the average of three replicated measurements.

The precision between the assays of amperometric measurements was tested with three measurements in 5 ng mL^{-1} P4 + 0.01 M PBS (pH 7.0). The percentage variation coefficient (%VC) was 4.5%, showing a good precision. The detection (DL) and the quantification (QL) limits were 0.2 and 0.5 ng mL^{-1}, respectively. The sensitivity was (3.0 ± 0.1) nA ng^{-1} mL in the range from 0.5 to 12.5 ng mL^{-1}.

The determination of P4 was carried out under the best experimental conditions found from bovine serum samples. Considering that serum samples did not contain P4, they were spiked with a known P4 concentration prepared in BPS 0.01 mol L^{-1} (pH 7.0) at a low buffer concentration (≤1%) in order to avoid a dilution effect. Therefore, two samples of serum containing 1 and 5 ng mL^{-1} of P4 were prepared. A given amount of P4–HRP was then added to both serum samples, with a dilution factor of 1/100 for P4 labeled to carry out competitive assays. Serum samples were introduced in the immunoreactor without any previous pretreatment. Obtained results are shown in Table 1, where current values represent an average of three replicated measurements, Recovery percentage values obtained were very good. Concentration values obtained by the ELISA method for the same samples are also shown in Table 1. The results obtained using both methods are very good.

The biosensor stability was tested for nearly 25 days at a P4 constant concentration in the inmunoreactor system. Current responses were practically constant during this period.

Table 1
Statistical analysis of inmunoreactor responses for two samples of serum with different amounts of spiked P4.

c^*_{P4} [a] (ng mL^{-1})	Current[b] (nA)	c^*_{P4} [c] (ng mL^{-1})	%VC[d]	Recovery (%)	c^*_{P4} [e] (ng mL^{-1})
1	50.0	1.03	4.02	103	0.995
5	38.1	5.10	5.80	102	4.86

[a] P4 concentration in samples of bovine serum.
[b] Current values represent an average of three measurements.
[c] Average value of P4 concentration determined by the immunosensor.
[d] Percentage variation coefficient.
[e] Average value of P4 concentration determined by ELISA method.

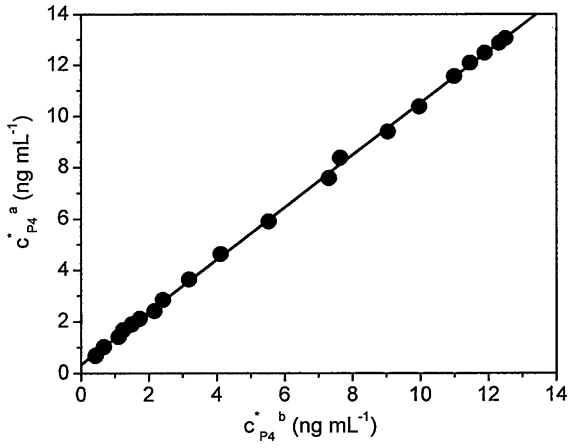


Fig. 5. Correlation between amperometric and commercial photometric assays. Slope = (1.019 ± 0.005), intercept = (0.34 ± 0.04) and r = 0,9997. (a) P4 concentration determined by ELISA method; (b) P4 concentration determined by the electrochemical immunosensor.

The immunoreactor was regenerated by injection of desorption buffer (0.1 mol L^{-1} glycine–HCl, pH 2.0) for 2 min and then washed with 0.01 mol L^{-1} in PBS (pH 7.0), which allowed us to use the reactor with about 100 determinations. For these measurements, P4 standard solutions as well as serum samples were employed alternatively. The reproducible current values that were found showed that the antibody activity loss was not observed. Then, the immunoreactor could be used for performing further analysis.

3.6. Correlations with ELISA assay procedure

The ELISA method was applied in accordance with the manufacturer's protocol. A calibration curve using the absorbance changes was constructed (figure not shown). The expression obtained using a least square procedure was:

$$\text{Absorbance} = (0.993 \pm 0.007) - (0.055 \pm 0.001)\,[\text{ng}^{-1}\,\text{mL}]\,c^{*}_{P4}\,[\text{ng mL}^{-1}] \quad r = 0.995$$

The %VC for the determination of 1 and 5 ng mL^{-1} P4 in bovine serum samples was 4.02 and 5.80% (three replicates), respectively. Results obtained by using the two methods are shown in Fig. 5.

Results of the linear correlation between the two methods render a slope of (1.019 ± 0.005) and an intercept of (0.34 ± 0.04) indicating a very good agreement between both methods (Fig. 5).

P4 ELISA assay kits for in-clinic uses allow P4 concentration to be determined either qualitatively or quantitatively. These results are usually obtainable within 45 to 60 min of sample collection. Most kits are modifications of kits marked for P4 assays used in the dairy industry [9]. The DL obtained for the ELISA procedure is similar to that determined with the electrochemical immunosensor (see Section 3.6). However, the ELISA method requires longer times of analysis. In a conventional ELISA test kit, 96 samples can run simultaneously. We did not compare the number of samples with this electroanalytical method but we just compared the general time of assays. This electroanalytical method could work with more immunosensors simultaneously. The determined number of samples analyzed with this methodology was greater than that obtained with the commercial ELISA test kits. Furthermore, ELISA test kit can be used only for one assay while the immunosensor we described can be used for up to 100 determinations.

Other electrochemical immunosensors to determine P4 in milk have been recently proposed [13–15]. Although the DL is comparable, most of them have higher variation coefficients (6–9%) as well as a longer analysis time. In addition, an important advantage of the immunosensor described in this paper is that it can perform up to 100 determinations before it is regenerated in a very simple way.

4. Conclusions

In this study an electrochemical immunosensor was developed to determine P4 at trace levels in the concentration range of interest in animal sera, particularly of bovine origin. These determinations performed without any pretreatment of samples would indicate the great selectivity of the antibody used. The immunoreactor developed can operate as a fast, selective, and sensitive detector when it is incorporated into a flow injection analysis system. The immunoreactor also minimizes the use of expensive antibodies and other reagents. It also shows physical and chemical stability, a wide working potential range and accuracy. It does not require highly skilled technicians or expensive and dedicated equipments. The electrochemical detection was carried out within 2 min and the total analysis time does not exceed 30 min. Moreover, the bonding between two biological molecules (P4 and HRP) was performed successfully, which allowed us to carry out competitive assays. Immunosensors based on specific reactions between antibodies and antigens seem to be promising alternative tools for the routine veterinary, clinical, biological, physiological assays and analytical practices.

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References

[1] C.A. Vijayavardhara, B. Halsall, W. Heineman, in: A. Brajter-Toth, J.Q. Chambers (Eds.), Electroanalytical Methods for Biological Materials, Marcel Dekker, New York, 2002.
[2] D. Hernández-Santos, M.B. González-García, A. Costa-García, Electroanalysis 14 (2002) 1225.
[3] P.L. Senger, Pathways to Pregnancy and Parturition, Current Conceptions, Inc., Pullman, USA, 1999.
[4] M.S. Christian, R.L. Brent, P. Calda, J. Mat-Fet. Neonat. Med. 20 (2007) 89.
[5] E. Ehrentreich-Forster, F.W. Scheller, F.F. Bier, Biosens. Bioelectron. 18 (2003) 375.
[6] L.E. McDonald (Ed.), Veterinary Endocrinology and Reproduction, 4th ed., Lea & Febinger, Philadelphia, London, 1989.
[7] P.J. Ball, Br. Vet. J. 138 (1982) 546.
[8] G. England, W. Concannon, Recent Advances in Small Animal Reproduction, International Veterinary Information Service, Ithaca, New York, USA, 2002, www.ivis.org.
[9] S.S. Deshpande, Enzyme Immunoassays, from Concept to Product Development, Chapman & Hall, New York, 1996.
[10] A.M.G. Bosch, H. Van Hell, J. Brands, R.H.W.M. Schuurs, in: S.P. Pal (Ed.), Proceedings of the International Symposium on Enzyme Labeled immunoassay of Hormones and Drugs, Walter de Gruyter, Berlin, 1978.
[11] V. Pucci, F. Bugamelli, R. Mandrioli, B. Luppi, M. Raggi, J. Pharm. Biomed. Anal. 30 (2003) 1549.
[12] J.P. Hart, R.M. Pemberton, R. Huxton, R. Wedge, Biosens. Bioelectron. 12 (1997) 1113.
[13] R.W. Claycomb, M.J. Delwiche, C.J. Munro, R.H. Bon Durant, Biosens. Bioelectron. 13 (1998) 1165.
[14] R.W. Claycomb, M.J. Delwiche, Biosens. Bioelectron. 13 (1998) 1173.
[15] V. Carralero, A. González-Cortés, P. Yánez-Seldeño, J.M. Pingarrón, Anal. Chim. Acta 596 (2007) 86.
[16] G. Gubitz, C. Shellum, Anal. Chim. Acta 283 (1993) 421.
[17] W.R. Heineman, H.B. Halsall, Anal. Chem. 57 (1985) 129.
[18] D. Athey, C.J. McNeil, J. Immunol. Methods 176 (1991) 153.
[19] S.S. Babkina, E.P. Medyantseva, H.C. Budnikob, M.P. Tyshlek, Anal. Chem. 68 (1996) 3827.

[20] K.R. Wehmeyer, H.R. Halsall, R. Heineman, C.P. Volle, I.W. Chen, Anal. Chem. 58 (1986) 135.
[21] J. Parellada, A. Narvaes, M.A. Lopez, E. Domínguez, J.J. Fernández, J. Katakis, Anal. Chim. Acta 362 (1998) 47.
[22] T. Lim, Y. Komoda, N. Nakamura, T. Matsunaga, Anal. Chem. 71 (1999) 1298.
[23] ABBOTT Diagnostics, Enzyme Immunoassay for the Quantitative Determination of Progesterone [Instruction Manual], Abbott Laboratories, Abbott Park, IL, U.S.A., 2009.
[24] L. Gorton, Electroanalysis 7 (1995) 23.
[25] C. Ruan, Y. Li, Talanta 54 (2001) 1095.
[26] N. Cénas, J. Rozgaite, A. Pocius, J.J. Kulys, J. Electroanal. Chem. 154 (1983) 121.
[27] L. Gorton (Ed.), Biosensors and Modern Biospecific Analytical Techniques, 44th ed., Elsevier, Amsterdam, Netherlands, 2005.
[28] G.A. Messina, A.J. Torriero, I.E. De Vito, J. Raba, Talanta 64 (2004) 1009.