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Ultrasensitive detection of adrenocorticotropin hormone (ACTH) using disposable phenylboronic-modified electrochemical immunosensors

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A B S T R A C T

This work reports for the first time an electrochemical immunosensor for the determination of adrenocorticotropin hormone (ACTH). The immunoelectrode design involves the use of amino phenylboronic acid for the oriented immobilization of anti-ACTH antibodies onto screen-printed carbon modified electrode surfaces. A competitive immunoassay between the antigen and the biotinylated hormone for the binding sites of the immobilized antibody was performed. The electroanalytical response was generated by using alkaline phosphatase-labelled streptavidin and 1-naphtyl phosphate as the enzyme substrate. The electrochemical oxidation of the enzyme reaction product, 1-naphtol, measured by differential pulse voltammetry was employed to monitor the affinity reaction. Under the optimized working conditions, an extremely low detection limit of 18 pg/L was obtained. Cross-reactivity was evaluated against other hormones (cortisol, estradiol, testosterone, progesterone, hGH and prolactin) and the obtained results demonstrated an excellent selectivity. The developed immunosensor was applied to a human serum sample containing a certified amount of ACTH with good results.

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1. Introduction

Human ~~adrenocontocotropin~~ adrenocorticotropin hormone (ACTH) is a peptide with 39 amino acids, 4500 Da, obtained by enzymatic cleavage of a precursor, the pro-opiomelanocortin (POMC) synthesized in the anterior pituitary (Yalow and Berson, 1971; Guillemin et al., 1977). The main function of the hormone is the induction of the synthesis of corticoids, especially cortisol. Levels of ACTH in serum fluctuate between <4.1 and 51.4 ng/L (Talbot et al., 2003) with the higher levels in the early morning and the lower levels in the evening (Norton et al., 2001). Several factors such as stress, pain, trauma, hypoxia, hypothermia and hypoglucemia can increase the concentration of ACTH in serum. The determination of ACTH levels in plasma is a key step in the diagnosis of pituitary Cushing's syndrome, adrenocortical insufficiency (Addinson's disease), hypopituitarism, and pituitary tumors (Talbot et al., 2003). Furthermore, the administration of ACTH is banned by the World Antidoping Agency (WADA) because it stimulates corticosteroid production and generates euphoria (Thevis et al., 2006). Various immunoassays have been proposed in the literature for the determination of ACTH using different antibodies and radioactivity, chemiluminiscence or fluorescence as detection techniques. These methods were claimed to be suitable to cover ranges between a few units up to various hundreds of ng/L ACTH (Talbot et al., 2003; Brossaud et al., 2011; Vogeser et al., 2000; Dobson et al., 1987). However, to our knowledge, no immunosensors for the determination of ACTH are described at present in the literature.

In particular, electrochemical immunosensors have demonstrated to gather useful analytical characteristics such high sensitivity, rapid response and easiness of use (Fowler et al., 2008). A crucial aspect in the design of these bioelectrodes is the combination of immobilization methods able to improve the immunoreagent stability and favourable orientation with a high electroanalytical performance. In this sense, various approaches have been described in the literature including the use of nanostructured electrode surfaces (Carralero et al., 2007; Wang, 2007; Pingarrón et al., 2008), self-assembled monolayers (Arya et al., 2009; Hao et al., 2007), or magnetic particles (Centi et al., 2007; Tang et al., 2010; Eguílaz et al., 2010). Another strategy that has demonstrated its usefulness for this purpose is the use of boronic acids which interact with sugars to form cyclic boronate esters at room temperature (Abad et al., 2002). This kind of binding reaction can be also successfully employed for the orientation of antibody molecules onto an electrode surface (Ho et al., 2011).

In this paper, we report the construction of an electrochemical immunosensor enabling the ultrasensitive determination of ACTH. The immunosensor design involves the combination of phenylboronic acid-modified screen-printed carbon electrodes,

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to efficiently immobilize ACTH antibodies, with a competitive immunoassay involving biotinylated ACTH, and a differential pulse voltammetric detection step using alkaline phosphatase-labelled streptavidin and 1-naphthyl phosphate (1-NPP) as enzyme substrate.

2. Experimental

2.1. Reagents and solutions

A 1.05 mg/mL stock solution of anti-ACTH C-terminal (Fitzgerald, MA, USA) was prepared by suitable dilution with 0.05 M phosphate buffer solution of pH 7.4 (PBS). Biotinylated ACTH (Biotin-ACTH) was obtained from AnaSpec, CA, USA, and 1.0 mg/mL solutions were prepared by dilution with distilled water. More diluted solutions were prepared by dilution with PBS. A stock 1.0 mg/mL alkaline phosphatase-labelled streptavidin (Strept-AP) (Sigma) solution was prepared in Tris–HCl buffer of pH 7.2, and more diluted solutions were prepared in the same buffer. A stock 1.0 mg/mL solution of ACTH from Genway Biotech, CA, USA was prepared in water and stored at −40 °C until use. More diluted solutions were prepared by dilution with PBS. 2% (w/v) bovine serum albumin (BSA, Type H2, Gerbu) solutions were prepared in PBS. A Trizma base (Sigma, 99%) and 1 mM magnesium chloride (Panreac, 99%) buffer solution of pH 9.0 (Trizma buffer), was also employed. 0.05 M solutions of 1-NPP (Fluka) were prepared by dilution with Trizma buffer. 4-Aminobenzoic acid (ABA, Across), 3-aminophenylboronic acid (APBA, Sigma), 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC, Sigma) and *N*-hydroxysuccinimide (NHS, Sigma), were also used. For the cross-reactivity assays, cortisol, testosterone, β-estradiol, progesterone, human growth hormone (hGH), all from Sigma, and prolactin (Immunometrics, UK), were tested.

All chemicals were of analytical-reagent grade and the water used was obtained from a Milli-Q purification system (Millipore, Bedford, NA, USA).

2.2. Samples

A certified sample of lyophilized human serum from NIBSC, 74/555, where each ampoule contained the freeze-dried residue of 0.5 mL of a solution which contained 25 μg ACTH was analyzed. The solid was reconstituted in distilled water for a final concentration of 10 μg/mL ACTH.

2.3. Apparatus and electrodes

All electrochemical measurements were carried out using a PGSTAT 12 potentiostat from Autolab. The electrochemical software was the general-purpose electrochemical system (GPES) (EcoChemie B.V.). Screen-printed carbon electrodes (4 mm diameter) were purchased from DropSens (Oviedo, Spain) and used as the working electrodes. These electrodes include a silver pseudo-reference electrode and a carbon counter electrode. All experiments were performed at room temperature. A P-Selecta ultrasonic bath, an Optic Ivymen System constant temperature incubator shaker (Comecta S.A.), and a P-Selecta Agimatic magnetic stirrer, all distributed by Scharlab, Madrid, Spain, were also used.

2.4. Procedures

2.4.1. Preparation of aminophenylboronic acid-modified screen-printed carbon electrodes (APBA–SPCEs)

Screen-printed carbon electrodes were modified with aminophenylboronic acid by means of a two step procedure. Firstly, 20 mg of ABA were dissolved in 2 mL of 1 M HCl and cooled with ice. Then, the diazonium salt was prepared by adding 2 mM $NaNO_2$ aqueous solution dropwise to this solution (38 μL for each 200 μL) with constant stirring. Next, 40 μL from the resulting solution was placed onto the SPCE and ten successive voltammetric cycles ranging between 0.0 and −1.0 V (υ = 200 mV/s) were carried out. The modified electrodes were washed thoroughly with water and methanol and dried at room temperature. In a second step, 10 μL from an EDC/NHS (0.1 M each) aqueous solution were placed onto the modified electrode and left to react for 1 h. After rinsing with water and methanol and drying, 10 μL from a 50 mM APBA were placed onto the electrode. After 3 h, the resulting aminophenylboronic electrode (APBA/SPCE) was rinsed with water and methanol, and dried.

2.4.2. Preparation of the ACTH immunosensor

10 μL of a 30 μg/mL anti-ACTH solution in PBS were dropped onto the APBA/SPCE and left to stand overnight. Then, 10 μL of a 2% BSA blocking solution were deposited onto the anti-ACTH/APBA/SPCE and left to incubate for 1 h. In order to perform the competitive assay, 10 μL of a mixture of the appropriate standard ACTH solution (or the sample) and 750 ng/mL Biotin-ACTH were placed onto the electrode surface and incubated for 45 min at 8 °C. Subsequently, 10 μL of 2000 ng/mL Strept-AP was added and, after 60 min, 45 μL of Trizma buffer plus 5 μL of 0.05 M 1-NPP solution were deposited onto the electrode and allowed to stand for 8 min. Differential pulse voltammograms were recorded over the −0.15 to +0.60 V potential range.

2.4.3. Sample analysis

Glass ampoules containing lyophilized serum samples were stored at −40 °C. When used, the solid from each ampoule was reconstituted in 2.5 mL distilled water at 20 °C by gentle stirring and mixing up to total dissolution. ACTH determination was performed by applying the procedure described above, and the peak current values measured by DPV were interpolated into the linear portion of the calibration plot obtained with ACTH standard solutions.

3. Results and discussion

The fundamentals of the developed immunosensor are illustrated in Fig. 1. Firstly, SPCEs were modified with boronic acid according to the procedure by Ho et al. (2011) with slight changes. Briefly, diazotation of ABA was performed with sodium nitrite in hydrochoride acid, the resulting 4-carboxybenzenediazonium ion solution was dropped onto the SPCE surface and the potential was cycled as described in Section 2. Fig. 2 shows the Nyquist plots obtained by electrochemical impedance spectroscopy using 5 mM $Fe(CN)_6^{4-}/Fe(CN)_6^{3-}$ as redox probe in 0.1 M KCl. As it can be seen, modification of the SPCE with ABA gave rise to a large increase in the electron transfer resistance (R_{CT} = 3050 Ω) as a consequence of the electrostatic repulsion between the redox probe and the negatively charged carboxylate groups. Thereafter, surface-confined carboxyl groups were activated with EDC/NHS and APBA moieties were attached to the electrode surface trough the formation of amide bonds. This modification with APBA produced a dramatic decrease in the R_{CT} value (307 Ω) due to the neutralization of the electrode negative surface charge. Then, anti-ACTH antibodies were attached to the resulting APBA/SPCE through the oriented boronic acid-saccharide bonds. After a blocking step with BSA, a competitive assay between ACTH and biotinylated ACTH for the binding sites of the immobilized antibodies was accomplished. Strept-AP was used to detect the immunosensing event by adding 1-NPP as AP substrate and measuring the electrochemical oxidation of the

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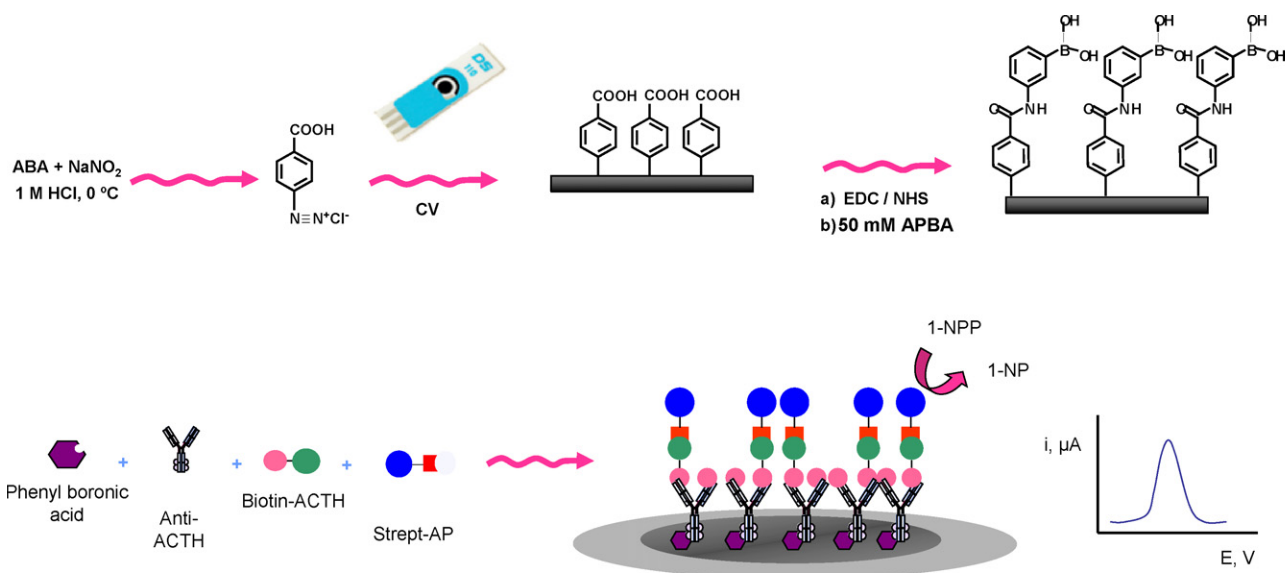

Fig. 1. Schematic display of the reactions and protocols involved in the development of disposable electrochemical immunosensors for ACTH using screen-printed electrodes modified with phenylboronic acid.

generated enzyme reaction product, 1-naphtol, by differential pulse voltammetry.

3.1. Optimization of the experimental variables involved in the immunosensor response to ACTH

All variables involved in the analytical performance of the immunosensor for ACTH were optimized. Firstly, the antibody loading onto the APBA/SPCE was evaluated by checking the voltammetric response obtained with different immunosensors, in the absence of ACTH, constructed upon deposition of 10 µL of anti-ACTH solution prepared in the 5–40 µg/mL antibody concentration range, and using 750 ng/mL Biotin-ACTH and 1 µg/mL Strept-AP. Fig. S1 (suppl. material) shows as the measured i_p value increased with the antibody loading up to 30 µg/mL reaching a saturated response for larger loadings. Therefore, 30 µg/mL anti-ACTH was selected for further work to attain the highest possible sensitivity. Anti-ACTH–APBA/SPCEs were allowed to stay overnight at 8 °C. In order to avoid non-specific binding of immunoreagents on the electrode surface (Wang et al., 2008), a blocking step with BSA was accomplished. Accordingly, both the BSA concentration and the incubation time employed to block the free sites on the electrode surface were also optimized. The protocol consisted of dropping 10 µL of 1, 2 or 5% BSA solutions, which were allowed to stand for 30, 60 or 120 min, onto the APBA/SPCEs and measuring the i_p values in the presence of 1500 ng/mL Biotin-ACTH and 2000 ng/mL Strept-AP. The obtained results (not shown) led us to choose a 2% BSA concentration and an incubation time of 60 min at room temperature as the optimum experimental conditions. Under these conditions unspecific responses were found to be always lower than 10% of the signals obtained with the Strept-AP–Biotin-ACTH-anti-ACTH–APBA/SPCE.

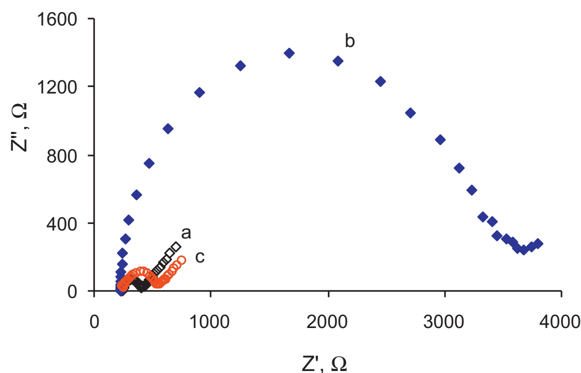

Fig. 2. Nyquist plots recorded from a 5 mM $[Fe(CN)_6]^{3-/4-}$ 0.1 M KCl solution at (a) bare SPCE, (b) 4-aminobenzoic acid-modified SPCE, (c) 3-aminophenylboronic acid-modified SPCE.

Another optimized variable was the concentration of Biotin-ACTH. In order to do that, different Strept-AP–Biotin-ACTH-anti-ACTH–APBA/SPCE immunosensors were prepared by incubating in Biotin-ACTH solutions with concentrations ranging in the 0–1500 ng/mL concentration range. The i_p values measured without ACTH showed an important increase from 0 to 750 ng/mL and a slight decrease for higher Biotin-ACTH concentrations (Fig. S2). Thus, 750 ng/mL was the selected value for further work. Moreover, the incubation time in the Biotin-ACTH solution was also evaluated in the 0 to 60 min interval by testing the voltammetric signal obtained with Strept-AP–Biotin-ACTH-anti-ACTH–APBA/SPCE immunosensors for 1 ng/mL ACTH. The largest peak current (results not shown) was obtained for 45 min and therefore, this was selected as the incubation time to be used. The effect of temperature in the incubation step was also investigated by recording DP voltammograms with immunosensors which were incubated at 8 °C, at room temperature, and at 38 °C. The obtained results (not shown) revealed that both the peak current and the reproducibility of the voltammetric responses decreased as the temperature increased, probably due to the lower stability of ACTH (Talbot et al., 2003). Thus, incubation in the Biotin-ACTH immunoreagent was accomplished at 8 °C.

In order to perform the competitive assay, Strept-AP was used. The conjugate concentration was optimized by testing the voltammetric response of different immunosensors incubated with Strept-AP in the 0 to 2000 ng/mL range. The obtained results (Fig. S3) revealed a regular current increase up to 1500 ng/mL whereas the peak current remained practically constant for larger conjugate concentrations. Therefore, 1500 ng/mL probably corresponded to saturation of the biotinylated ACTH with Strept-AP. Accordingly, in order to achieve the optimal experimental conditions to detect the competition between ACTH and Biotin-ACTH

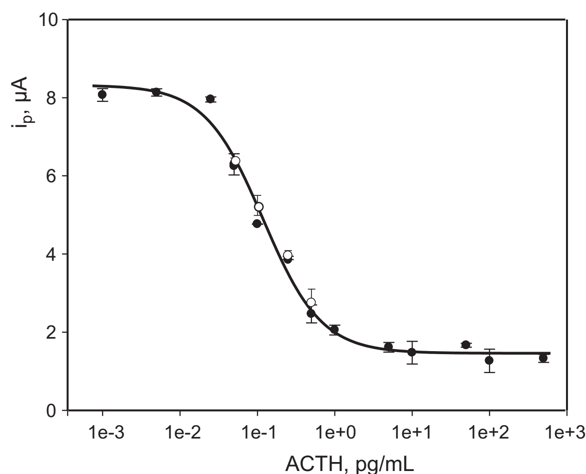

Fig. 3. Calibration plot for ACTH obtained with the Strept-AP–Biotin-ACTH-anti-ACTH–APBA/SPCE immunosensor. Open points correspond to the serum sample analysis.

for the anti-ACTH binding sites, a concentration of 2000 ng/mL Strept-AP was selected. Moreover, the incubation step was found to provide optimum results when it was carried out at 8 °C for 60 min (results not shown).

Furthermore, the voltammetric behaviour at the SPCE of different phenolic compounds (indoxyl, 4-aminophenol and 1-naphtol), which can be generated as alkaline phosphatase reaction products from their respective phosphate derivatives used as enzyme substrates, was checked. Cyclic voltammograms from 5.0 mM solutions of these phenolic compounds in Trizma buffer of pH 9.0 (results not shown) showed that the 1-naphthol irreversible oxidation reaction occurred at a remarkably less positive potential than those observed for the other compounds. Although the peak current measured for 1-naphthol was slightly lower than that obtained for the other phenolics, as a compromise between sensitivity and selectivity, 1-NPP was selected as the enzyme substrate to be used in this work. The influence of 1-NPP concentration on the differential pulse voltammetric responses of the Strept-AP–Biotin-ACTH-anti-ACTH–APBA/SPCE immunosensor is shown in Fig. S4. As it can be seen, the highest peak current value was obtained at 5 mM 1-NPP, which represents a relatively high concentration then ensuring that the enzyme reaction rate depended only on the enzyme concentration.

3.2. Analytical characteristics of the immunosensor for ACTH

Fig. 3 shows the calibration plot constructed for ACTH under the optimized working conditions stated above. Error bars displayed were calculated from measurements carried out with three different immunosensors, except in the case of the points corresponding to the absence of ACTH and in the EC_{50} value, where eight measurements were made (see below). The calculated errors were below 10% in all cases. The ACTH concentration tested ranged between 10^{-3} and 500 pg/mL ACTH. The i_p vs. ACTH concentration curve was fitted by non-linear regression using the Sigma Plot data analysis software (Coneely et al., 2007). The adjusted equation (r = 0.995) was:

$$i_p = \frac{i_{max} - i_{min}}{1 + EC_{50}/x} + i_{min}$$

where i_{max} and i_{min} were the maximum and minimum peak current values of the calibration graph: 8.3 ± 0.2 μA and 1.5 ± 0.2 μA, respectively. The EC_{50} value, which is the ACTH concentration corresponding to a fifty per cent competition, was 0.10 ± 0.02 pg/mL,

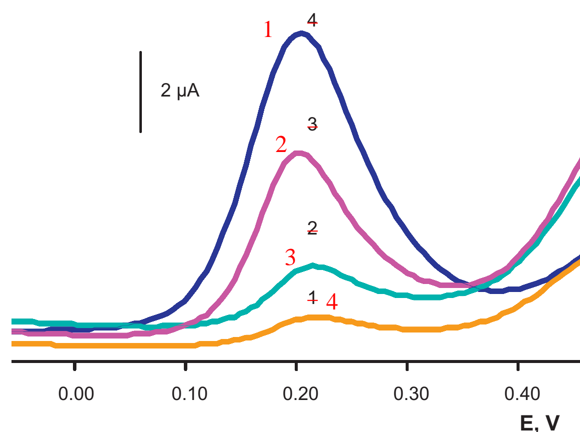

Fig. 4. Differential pulse voltammograms recorded with the Strept-AP–Biotin-ACTH-anti-ACTH–APBA/SPCE immunosensor for 0 (1), 0.05 (2), 0.50 (3), and 1.00 (4) pg/mL ACTH.

and the Hill slope, h, was −1.16 ± 0.15. The observed range of linearity ranged between 0.025 and 1.0 pg/mL (r = 0.990), which is adequate for the analysis of clinical samples. A very low limit of detection, 18 pg/L ACTH was calculated from the equation:

$$\text{LOD} = \text{EC}_{50}\left(\frac{i_{max} - i_{min}}{i_{max} - i_{min} - 3s} - 1\right)^{-1/h}$$

where s is the standard deviation (n = 10) of the zero value (the i_p value measured in the absence of ACTH), ±0.28 μA.

As an example of the voltammetric curves recorded, Fig. 4 displays the responses obtained for 0, 0.05, 0.50 and 1.00 pg/mL ACTH. It can be observed as concentrations of the hormone as low as 0.05 pg/mL yielded very good DP voltammetric signals.

The reproducibility of the DP voltammetric measurements was evaluated by carrying out assays with different immunosensors both for solutions containing no ACTH and for a 0.10 pg/mL (the EC_{50} value) ACTH concentration level on the same day and on different days. Relative standard deviation (RSD) values of 3.3 and 4.4% (n = 8) were obtained, respectively, for the assays performed on the same day, whereas the RSD values (n = 8) were 8.9% when no ACTH was present and 5.9% for 0.10 pg/mL ACTH when the measurements were carried out on different days. The storage stability of the APBA/SPCE modified electrodes, as well as that of anti-ACTH–APBA/SPCE and Strept-AP–Biotin-ACTH-anti-ACTH–APBA/SPCE immunosensors was also investigated. In order to do that, the different modified electrodes and immunosensors were prepared on the same day and stored in a refrigerator at 4 °C. Then, every electrode and bioelectrode was used to construct the corresponding immunosensor and to measure the voltammetric response for 5 mM 1-NPP in the absence of ACTH. Fig. 5 shows the results obtained during a period of time of 27 days for APBA/SPCE modified electrodes (●) and anti-ACTH–APBA/SPCE

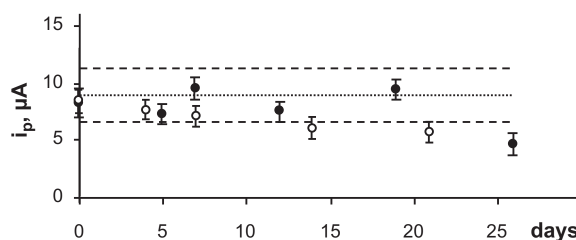

Fig. 5. Control chart constructed for APBA/SPCE (●) and anti-ACTH–APBA/SPCE (○). Each point corresponds to the mean value for three successive measurements of 5 mM 1-NPP.

Table 1
Cross-reactivity calculated from the EC_{50} values for different hormones with the Strept-AP–Biotin-ACTH-anti-ACTH–APBA/SPCE immunosensor.

Compound	EC_{50} (ng/mL)	CR[a] (%)
ACTH	1.0×10^{-4}	100
Cortisol	0.47	0.02
Prolactin	1.11	0.01
Estradiol	13,733	7.3×10^{-7}
Testosterone	>20,000	$<5.0 \times 10^{-7}$
Progesterone	>20,000	$<5.0 \times 10^{-7}$
hGH	>20,000	$<5.0 \times 10^{-7}$

[a] %CR = 100 (EC_{50}) ACTH/(EC_{50}) interfering compound.

immunosensors (○). Each point in the displayed control chart corresponded to the mean value of three successive measurements. As it can be seen, the APBA/SPCE modified electrodes yielded voltammetric responses that remained inside the control limits set at ±3 s calculated for the whole series of experiments, for at least 18 days. However, a 63% of the initial current was observed on the 27th day. On the other hand, the DPV responses measured with anti-ACTH–APBA/SPCE immunosensors remained inside the control limits for 15 days and the current measured on the 21st day corresponded to 83% of the initial value. Regarding the bioelectrode containing all the bioconjugates used in the immunosensor functioning, Strept-AP–Biotin-ACTH-anti-ACTH–APBA/SPCE, its storage stability was much poorer retaining only a 30% of the initial value after five days from its preparation.

3.3. *Cross-reactivity*

Various hormones (cortisol, testosterone, β-estradiol, progesterone, human growth hormone (hGH), and prolactin) which may be present in biological fluids together with ACTH, were tested as potential interfering substances for the immunosensor response towards ACTH. The cross-reactivity was calculated from the EC_{50} value for each tested compound and the obtained results are summarized in Table 1. As it can be observed, all the tested species gave very low % CR, with values lower than 0.01% except in the case of cortisol (0.02%). These results demonstrated the high selectivity of the developed immunosensor.

3.4. *Determination of ACTH in certified serum samples*

The usefulness of the immunosensor for the analysis of real samples was checked by analyzing a certified human serum sample. Calibration graphs were constructed from the reconstituted sample which was appropriately diluted to obtain 0.05, 0.10, 0.25 or 0.50 pg/mL ACTH. Fig. 3 shows this calibration graph superimposed on the calibration curve for ACTH obtained with standards. Errors bars were calculated in all cases for three different measurements. As it can be observed, the analytical responses overlapped completely in both calibration plots, with a linear portion for the calibration recorded with the serum sample exhibiting a slope value of −1.54 μA (r = 0.997) which was very similar to that obtained for ACTH standard solutions (−1.52 μA; r = 0.990). Therefore, the determination of ACTH could be accomplished by interpolation of the measured ip values into the calibration plot obtained with ACTH standard solutions. The results obtained for the analyses carried out at the four concentration levels mentioned above were 0.050 ± 0.001, 0.11 ± 0.02, 0.23 ± 0.02 and 0.49 ± 0.015 pg/mL ACTH for five replicates, with recoveries ranging between 92 ± 3 and 106 ± 4%. These results demonstrated the usefulness of the developed immunosensor for the analysis of low ACTH concentrations in human sera with practically no sample treatment.

4. Conclusions

In this paper, the development of an electrochemical immunosensor for ACTH is reported for the first time. The immunosensing approach involves the immobilization of anti-ACTH onto phenylboronic acid-modified screen-printed carbon electrodes and a competitive immunoassay implying biotinylated ACTH and alkaline phosphatase labelled streptavidin. Moreover, a differential pulse voltammetric analytical response was used to monitor the affinity reaction. The developed design exhibits an excellent analytical performance in terms of sensitivity, selectivity, wide range of quantifiable antigen concentrations, and inter-assay reproducibility. Therefore, this immunosensor can be envisaged as a useful and affordable analytical tool for the rapid determination of ACTH in clinical applications, as it has been outlined with the results obtained for the analysis of certified human serum samples.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2012.02.015.

References

Abad, J., Vélez, M., Santamaría, C., Guisan, J., Matheus, P., Vázquez, L., Gazaryan, I., Gorton, L., Gibson, T., Fernández, V., 2002. J. Am. Chem. Soc. 124, 12845–12853.
Arya, S.K., Solankia, P.R., Datta, M., Malhotra, D.B., 2009. Biosens. Bioelectron. 24, 2810–2817.
Brossaud, J., Bouton, M., Gatta, B., Tabarin, A., Corcuff, J.B., 2011. Clin. Biochem. 44, 1160–1162.
Carralero, V., González-Cortés, A., Yáñez-Sedeño, P., Pingarrón, J.M., 2007. Anal. Chim. Acta 596, 86–91.
Centi, S., Laschi, S., Mascini, M., 2007. Talanta 73, 394–399.
Coneely, G., Aherne, M., Lu, H., Guilbault, G.G., 2007. Anal. Chim. Acta 583, 153–160.
Dobson, S., White, A., Hoadley, M., Lovgren, T., Ratcliffe, J., 1987. Clin. Chem. 33, 1747–1751.
Eguílaz, M., Moreno-Guzmán, M., Campuzano, S., González-Cortés, A., Yáñez-Sedeño, P., Pingarrón, J.M., 2010. Biosens. Bioelectron. 26, 517–522.
Fowler, J.M., Wong, D.K.Y., Halsall, H.B., Heineman, W.R., 2008. In: Zhang, X., Ju, H., Wang, J. (Eds.), Recent Developments in Electrochemical Immunoassays and Immunosensors. Academic Press, pp. 115–143, Ch. 5.
Guillemin, R., Vargo, T., Rossier, J., Minick, S., Ling, N., Rivier, C., Val, W., Bloom, F., 1977. Science 197, 1367–1369.
Hao, C., Yan, F., Ding, L., Xue, Y., Ju, H., 2007. Electrochem. Commun. 9, 1359–1364.
Ho, J.A., Hsu, W.A., Liao, W.C., Chiu, J.K., Chen, M.L., Chang, H.C., Li, C.C., 2011. Biosens. Bioelectron. 26, 1021–1027.
Norton, J.A., Li, M., Gillary, J., Le, H.N., 2001. Curr. Prob. Surgery 38, 448–545.
Pingarrón, J.M., Yáñez-Sedeño, P., González-Cortés, A., 2008. Electrochim. Acta 53, 5848–5866.
Talbot, J.A., Kane, J.W., White, A., 2003. Ann. Clin. Biochem. 40, 453–471.
Tang, D.P., Su, B.L., Tang, J., Ren, J., Chen, G.N., 2010. Anal. Chem. 82, 1527–1534.
Thevis, M., Bredehöft, M., Geyer, H., Kamber, M., Delahaut, P., Schänzer, W., 2006. Rapid Commun. Mass Spectrom. 20, 3551–3556.
Vogeser, M., Engelhardt, D., Jacob, K., 2000. Clin. Chem. 46, 1998–2000.
Wang, Z., Tu, Y., Liu, A., 2008. Talanta 77, 815–821.
Wang, J., 2007. Electroanalysis 19, 769–776.
Yalow, R.S., Berson, S.A., 1971. Biophys. Res. Commun. 44, 439–445.

Fig. S1. Effect of the immobilized anti-ACTH loading on the DPV peak current measured with Strept-AP–Biotin-ACTH-anti-ACTH–APBA/SPCE immunosensors. See text for other conditions.

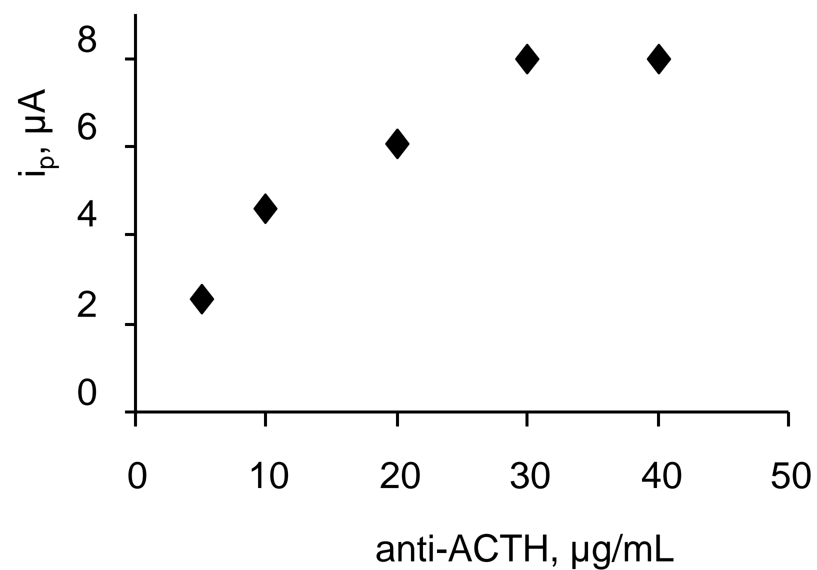

Figure S1

Fig. S2. Effect of the Biotin-ACTH concentration on the DPV peak current measured with Strept-AP–Biotin-ACTH-anti-ACTH–APBA/SPCE immunosensors. See text for other conditions.

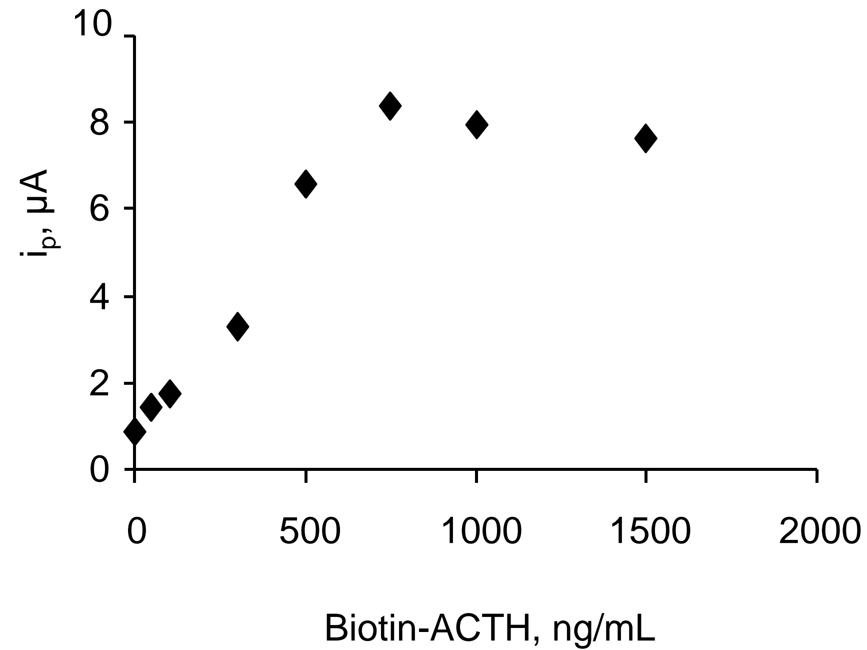

Figure S2

Fig. S3. Effect of the Streptavidin-AP concentration on the DPV peak current measured with Strept-AP–Biotin-ACTH-anti-ACTH–APBA/SPCE immunosensors. See text for other conditions.

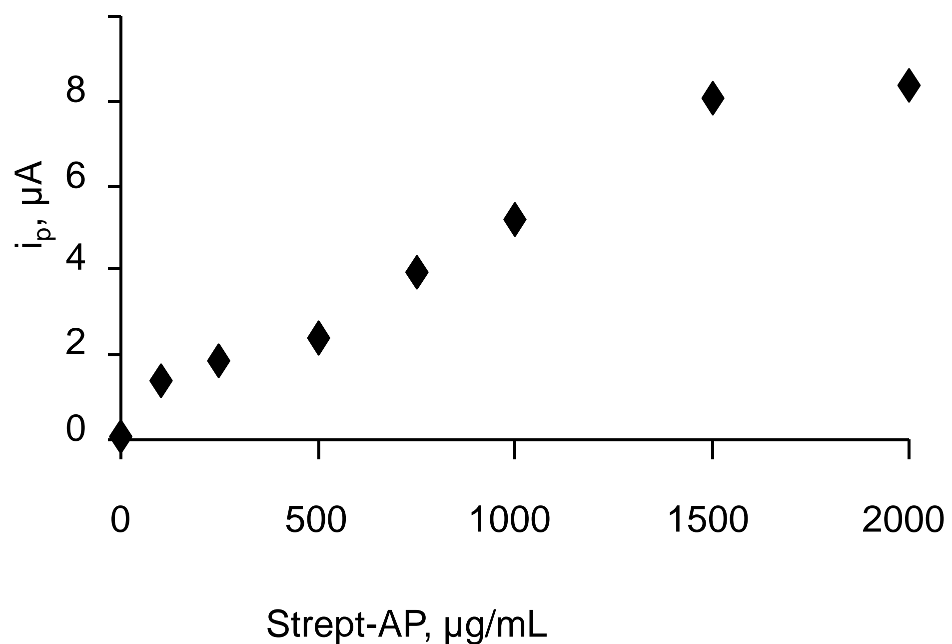

Figure S3

Fig. S4. Effect of the 1 NPP concentration on the DPV peak current measured with Strept-AP–Biotin-ACTH-anti-ACTH–APBA/SPCE immunosensors. See text for other conditions.

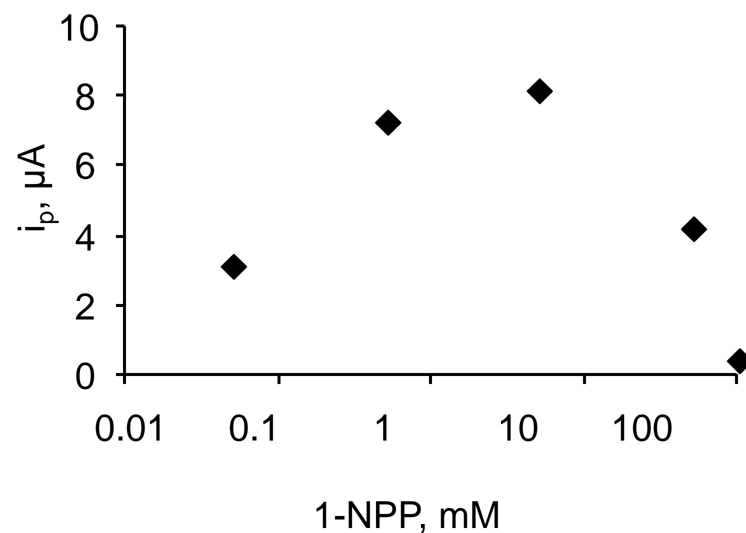

Figure S4

Fig. S1. Effect of the immobilized anti-ACTH loading on the DPV peak current measured with Strept-AP–Biotin-ACTH-anti-ACTH–APBA/SPCE immunosensors. See text for other conditions.

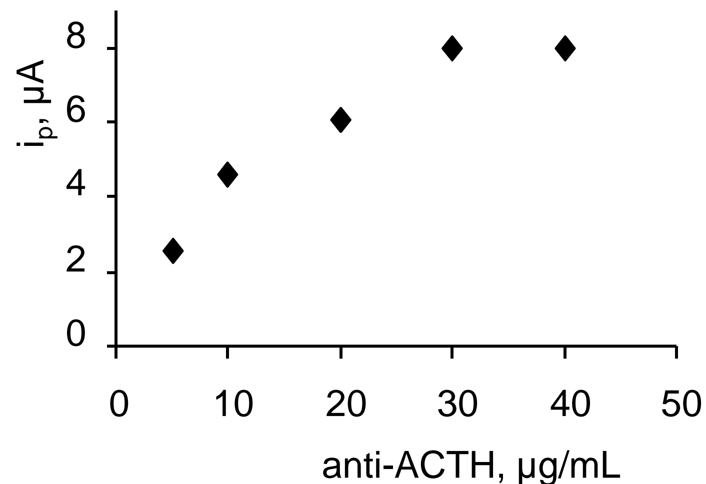

Fig. S2. Effect of the Biotin-ACTH concentration on the DPV peak current measured with Strept-AP–Biotin-ACTH-anti-ACTH–APBA/SPCE immunosensors. See text for other conditions.

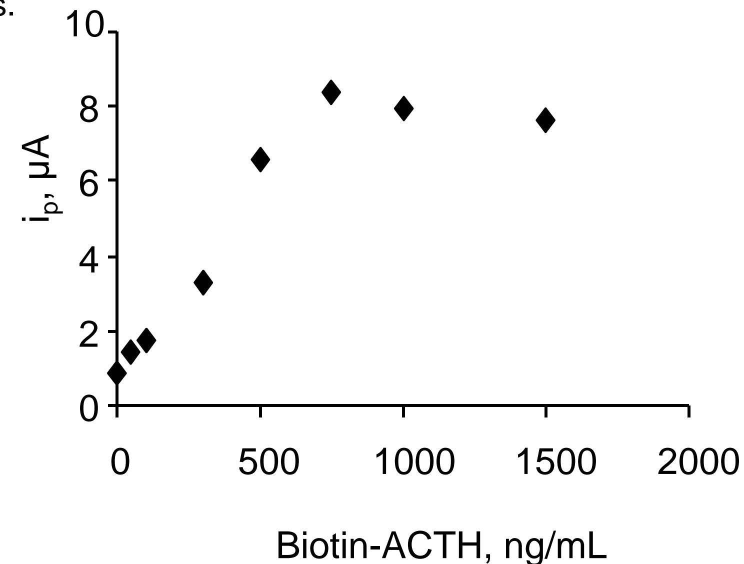

Fig. S3. Effect of the Streptavidin-AP concentration on the DPV peak current measured with Strept-AP–Biotin-ACTH-anti-ACTH–APBA/SPCE immunosensors. See text for other conditions.

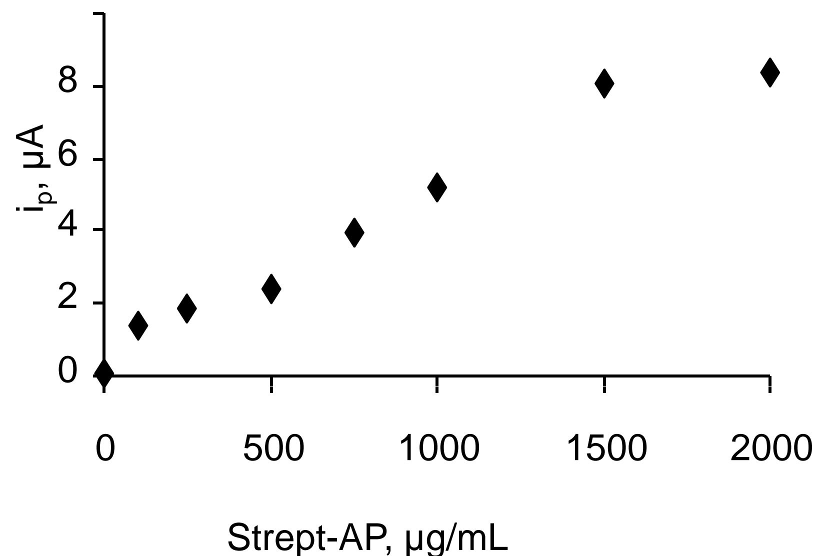

Fig. S4. Effect of the 1 NPP concentration on the DPV peak current measured with Strept-AP–Biotin-ACTH-anti-ACTH–APBA/SPCE immunosensors. See text for other conditions.