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A novel sensitive electrochemical DNA biosensor for assaying of anticancer drug leuprolide and its adsorptive stripping voltammetric determination

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ABSTRACT

The anticancer drug, leuprolide (LPR) bound to double-stranded fish sperm DNA (dsDNA) which was immobilized onto the surface of an anodically activated pencil graphite electrode (PGE), was employed for designing a sensitive biosensor. The interaction of leuprolide (LPR) with double-stranded DNA (dsDNA) immobilized onto pencil graphite electrode (PGE) have been studied by electrochemical methods. The mechanism of the interaction was investigated and confirmed by differential pulse voltammetry using two different interaction methods; at the PGE surface and in the solution phase. The decrease in the guanine oxidation peak current was used as an indicator for the interaction in acetate buffer at pH 4.80. The response was optimized with respect to accumulation time, potential, drug concentration, and reproducibility for both interaction methods. The linear response was obtained in the range of 0.20–6.00 ppm LPR concentration with a detection limit of 0.06 ppm on DNA modified PGE and between 0.20 and 1.00 ppm concentration range with detection limit of 0.04 ppm for interaction in solution phase method. LPR showed an irreversible oxidation behavior at all investigated pH values on a bare PGE. Differential pulse adsorptive stripping (AdSDPV) voltammetric method was developed for the determination of LPR. Under these conditions, the current showed a linear dependence with concentration within a range of 0.005–0.20 ppm with a detection limit of 0.0014 ppm. Each determination method was fully validated and applied for the analysis of LPR in its pharmaceutical dosage form.

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1. Introduction

Electrochemical biosensors have played an important role in the transition towards point-of-care diagnostic devices [1]. After the discovery of electroactivity in nucleic acids at the beginning of the sixties [2], many electrochemical approaches have been performed for the analysis of nucleic acids. Nucleic acid layers combined with electrochemical transducers produce a new kind of affinity biosensor for nanomolecules [3]. The attractive properties of electrochemical devices are extremely promising for improving the efficiency of cancer diagnostics and therapy monitoring [4]. With further developments and resources, these devices provide quick analytical results available at patient's bedside or physician's office within a few minutes [1].

Electrochemical investigation of DNA–drug interactions can provide a rapid, sensitive, selective and cheap method for the determination of antineoplastic drugs. Electrochemical DNA biosensors, especially using disposable PGEs are assayed more easily and rapidly compared to the conventional DNA biosensors. The single use pencil graphite electrode for electrochemical DNA biosensors have several advantages, such as avoidance of contamination among samples, ease of use because without the need of pretreatment and constant sensitivity, selectivity and reproducibility [1–4]. Electrochemical approach can provide new insights into rational drug design and will lead to further understanding of the mechanism of interaction between anticancer drugs and DNA [4]. Electrochemical biosensing micro-system for the rapid point-of-care genetic screening of breast cancer has been developed [5].

LPR (Scheme 1) is a gonadotropin-releasing hormone (GnRH) agonist. By causing constant stimulation of the pituitary GnRH receptors, LPR initially causes stimulation, but thereafter decreases pituitary secretion of the luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LPR may be used in the treatment of hormone-responsive cancers such as breast cancer or prostate cancer, estrogen-dependent conditions such as endometriosis [6] or uterine fibroids, as well as to treat precocious puberty [7], and to control ovarian stimulation during in vitro fertilization procedures [8].

The widespread use of LPR and the need for clinical and pharmacological study require fast and sensitive analytical techniques to assay the presence of the drug in pharmaceutical dosage forms and biological samples. LPR has only been studied and analyzed by chromatographic techniques: HPLC [9] and LC–MS [10–13]. The reported HPLC and LC–MS methods were influenced by the inter-

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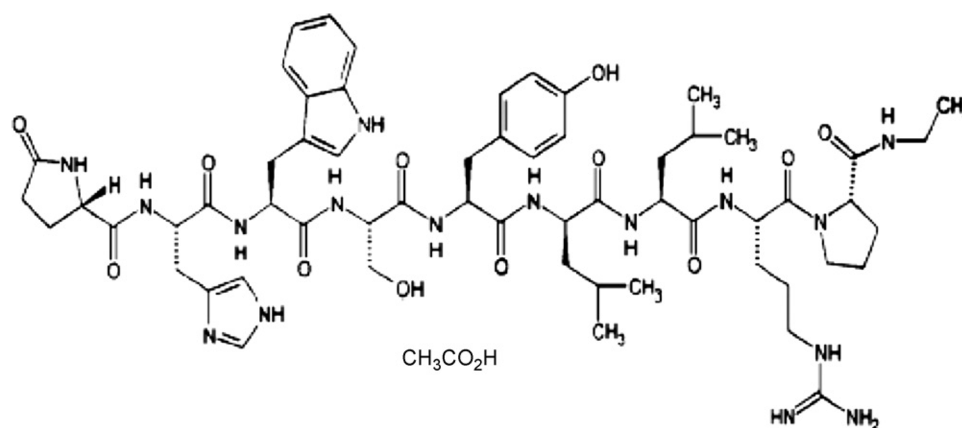

Scheme 1. The chemical structure of LPR.

ference of endogenous substances and potential loss of drugs in the re-extraction procedure, and involved tedious and time-consuming plasma sample preparation and extraction processes.

Electroanalytical methods are well known for their high sensitivity, and these techniques have been used for the analysis of pharmaceuticals due to their sensitivity, selectivity, simplicity, low cost, and relatively short analysis time when compared with the other techniques [14–16]. Electrochemical techniques also help for the identification of the redox mechanism of drug compounds and provide important information about DNA–drug interactions [17–20]. Disposable PGE (pencil graphite electrode) for DNA biosensors has several advantages, such as prevention of sample cross-contamination and loss of response due to electrode impurity [21–23]. Hence, the electrochemical DNA-biosensor enables to evaluate and predict DNA interactions and damage by health hazardous compounds based on their binding to nucleic acids [24]. This information is valuable in drug discovery and can speed up the investigation of new pharmaceutically active compounds.

Electrochemical detection of the interaction between LPR and DNA based on the changes of guanine signal has not been studied on any electrode before. This study does not only report electrochemical DNA interactions of LPR but also electrochemical determination of LPR alone. Furthermore, there appears to be no sensitive analytical method for the determination of LPR either in bulk form or pharmaceutical dosage forms that have been reported up-to-date.

Accordingly, our aim is to develop a new, sensitive and selective electrochemical DNA biosensor for the detection and the determination of leuprolide (LPR). In this study, two different electrochemical interaction techniques between double-stranded fish sperm DNA (ds-DNA) and LPR are investigated. The current study is broadly divided into three sections. The first part involves the detection and determination of ds-DNA–LPR interaction on PGE based on the changes of guanine signal using differential pulse voltammetry. Secondly, special attention will be given for the confirmation and proving of this interaction measuring after interaction in the solution phase between ds-DNA and LPR on bare PGE. Finally, the third part involves cyclic and adsorptive stripping differential pulse voltammetric (AdSDPV) studies for identification of electrochemical oxidation behavior and the selective, sensitive and fully validated determination of LPR using bare PGE.

2. Experimental

2.1. Apparatus

Voltammograms were recorded using an AUTOLAB-PGSTAT 30 electrochemical analysis system that was monitored with a personal computer using General Purpose Electrochemical Software (GPES) 4.9 software (Eco Chemie, Utrecht, The Netherlands). A conventional three-electrode cell with an Ag/AgCl (BAS; 3 M KCl) reference, a Pt wire counter and the PGE as the working electrodes were used. The disposable PGE (Tombo Japan) that was adopted in the study by Wang et al. [25], used in all experiments. A Rotring® pencil Model Tikky II (Germany) was used as a holder for the graphite lead. The details of this technique were presented in our previous paper [26].

pH was measured using a pH meter Model 538 (WTW, Austria) using a combined electrode (glass electrode–reference electrode) with an accuracy of pH ± 0.05.

DPV conditions were given as follows—step potential: 0.00795 V; modulation amplitude: 0.0505 V; modulation time: 0.05 s; interval time: 0.5 s. Cyclic voltammetric measurements were also realized by using AUTOLAB-PGSTAT 30 system. The accumulation potential (700 mV) for the electroanalytical studies was applied for a selected deposit time (180 s). Electrochemical cell solution was stirred at 350 rpm during the accumulation step.

The raw voltammograms of DPV technique were used after treatment with General Purpose Electrochemical Software (GPES 4.9). Average baseline correction defined as in the literature [27] using a ‘peak width’ of 0.01 V.

Each cyclic or differential pulse voltammetric measurements were performed using a new and same size PGE without any pretreatment procedure.

2.2. Chemicals

ds-DNA was obtained from Serva Company (Germany). LPR and its pharmaceutical dosage form were kindly supplied by Abbott Pharmaceutical Company (Istanbul, Turkey). All other chemicals for the preparation of buffers and supporting electrolytes were reagent grade (Merck or Sigma). The ds-DNA stock solution (10 mg/10 mL) was prepared with ultrapure water and kept frozen (−20 °C). More diluted solution of ds-DNA were prepared with 0.50 M acetate buffer solution (pH 4.80) containing 0.02 M NaCl. All working solutions were prepared using analytical grade reagents and purified water from a Millipore Milli-Q system.

Stock solutions of LPR (500 ppm) were prepared in pure water and kept in the dark in a refrigerator. For the voltammetric studies, Britton–Robinson buffer (0.04 M, pH 2.00–12.00) were prepared in double-distilled water. All experiments were carried out at room temperature. The diluted solutions were prepared daily by accurate dilution with the selected supporting electrolyte just before use and protected from light to avoid the degradation. All freshly

prepared diluted solutions, were stable for at least 10 h. The ruggedness, within day and between day precisions were checked and results were given as relative standard deviation (RSD%) values [28–30]. All necessary validation parameters were calculated and reported.

2.3. Adsorptive stripping voltammetric assay

Adsorptive stripping differential pulse voltammetric (AdSDPV) analyses were carried out in Britton–Robinson buffer at pH 2.00 on bare PGE for LPR alone. The required aliquot of LPR working solutions were placed in the voltammetric cell containing the selected supporting electrolyte. The bare PGE was kept in the cell. The accumulation potential (usually open circuit condition) was applied for a selected deposit time (180 s) while the solution was stirred at 350 rpm. The stirrer was then stopped and after 10 s rest period, the compound was removed by stripping anodically using DPV method. Operating conditions were as described in Section 2.1.

2.4. Interaction of LPR with ds-DNA

2.4.1. Procedure for the ds-DNA modified PGE for the DNA–drug interaction studies

PGE surface was pretreated by applying +1.40 V for 60 s in 0.50 M acetate buffer solution containing 0.02 M NaCl (pH 4.80) without stirring, creating a surface on which DNA was adsorbed through electrostatic interaction with carboxyl moieties [31]. The ds-DNA was immobilized on a pretreated PGE by applying a potential at +0.50 V during 240 s using 400 rpm stirring rate in 2.00 ppm ds-DNA in 0.50 M acetate buffer (pH 4.80) solution containing 0.02 M NaCl. The electrode was then gently rinsed with acetate buffer solution (0.50 M at pH 4.80) for 2 s for the removal of the unbound ds-DNA at the electrode surface. The ds-DNA modified PGE was immersed in the blank 0.50 M acetate buffer solution (pH 4.80) containing 0.02 M NaCl and differential pulse voltammograms were recorded in the range of +0.40 to +1.40 V until stabilization of the peak currents that correspond to guanine electrooxidation occurred. The procedure was repeated by using a new PGE. After immobilization of ds-DNA and rinse period, the ds-DNA modified PGE was immersed into 0.50 M acetate buffer solution (pH 4.80) with different concentrations of LPR with 400 rpm stirring for 90 s at open circuit system. The electrode was then rinsed with 0.50 M acetate buffer solution for 2 s.

The oxidation signals of guanine, before and after interaction with drug, were taken using DPV mode in the blank 0.50 M acetate buffer solution at pH 4.80 containing 0.02 M NaCl.

2.4.2. Procedure for the interaction between ds-DNA and LPR in the solution phase

PGE was pretreated by applying +1.40 V for 60 s in acetate buffer solution without stirring for the activation. The ds-DNA was immobilized on a pretreated PGE by applying open circuit system for 120 s using 400 rpm stirring rate in 5.00 ppm ds-DNA (in 0.50 M acetate buffer solution containing 0.02 M NaCl, pH 4.80). The electrode was then gently rinsed with acetate buffer solution for 2 s. The ds-DNA attached PGE was immersed in the blank acetate buffer and differential pulse voltammograms were recorded in the range of +0.40 to +1.40 V until stabilization of the peak currents that correspond to guanine electrooxidation occurred. Then, the procedure was repeated by using a new PGE, for ds-DNA–LPR interaction in solution phase. After the PGE electrode activation step, the standard addition methods were performed to ds-DNA solution for the interaction. Progressively, different LPR concentrations were added to the previous 5.00 ppm ds-DNA solution, containing 0.02 M NaCl (pH 4.80) and stirred at 400 rpm rate for 120 s at +0.00 V. Following a gentle washing process in acetate buffer, the electrode was transferred into a voltammetric cell containing pH 4.80 acetate buffer.

The oxidation signals of guanine, before and after interaction with the drug, were taken using DPV mode. The obtained curves from before and after interactions between drug and ds-DNA were compared with each other.

2.5. Injection dosage form assay procedure

Adequate amount of Lucrin® injectable solution, claim to contain 5.00 mg LPR per 1 mL of the solution, was dissolved in 10 mL pure water. The inactive ingredients present in Lucrin® injectable dosage form were purified gelatin, poly (DL-lactic acid) co-glycolic acid copolymer, D-mannitol, carboxy methyl cellulose sodium, polysorbate 80.

An aliquot of this solution was transferred into a 10 mL volumetric flask, diluted to the volume with supporting electrolyte and the voltammogram was recorded.

The nominal content of the injectable solution was calculated from the corresponding regression equations of previously plotted calibration plots obtained using PGE electrode.

3. Results and discussion

The electrochemical oxidation of nucleic acids at pH 4.80 is due to the oxidation of the purine residues [32], guanine and adenine, in the polynucleotide chains. The evaluation of any interaction with DNA using biosensors helps to predict unwanted toxic side-effects and prevent DNA damage caused by therapeutic drugs [33]. Our proposed study is the first work for the ds-DNA–LPR interaction and the detailed electrochemical behavior of LPR on bare PGE. In this study, the experimental conditions such as LPR concentration, ds-DNA concentration and interaction time between LPR and ds-DNA and the effect of the ionic strength are studied as detailed.

3.1. Electrochemical investigation of LPR at bare PGE

To demonstrate the usefulness of a solid electrode for the determination of LPR which may offer advantages for the use of such electrodes as sensors, the electrochemical behavior of LPR on PGE was investigated in this study. As a first step, LPR was subjected to cyclic voltammetric studies with the aim of characterizing its electrochemical behavior in different pH values. As the second step, LPR was subjected to a voltammetric determination with the AdSDPV mode using bare PGE. As seen in Fig. 1a, the cyclic voltammograms of 4.00 ppm LPR, showed a sharp and well-defined anodic peak (Ox_1) in Britton–Robinson buffer at pH 2.00 using 100 mV s^{-1} scan rate on the PGE. This anodic peak appeared at about 0.92 V in the initial anodic scan (Fig. 1a). Depending on pH, LPR oxidation peak was splitted (Fig. 1b and c). After pH 10.00, this peak nearly becomes independent from pH. Upon scan reversal, no corresponding reduction peak was obtained to the anodic peak on the cathodic branch. However, one small reduction peak appeared on cathodic branch at about 0.35 V. On the second positive sweep, two additional anodic peaks appeared at less positive potential values than that of the drug, namely Ox_2 and Ox_3, at about 0.40 V and 0.82 V, respectively. These additional anodic peaks only appeared on the second and further cycles of oxidation. It may be suggested that some chemical follow-up reaction had occurred at the initial charge transfer, thus Ox_2 and Red_1 form obtained as a new reversible redox couple and Ox_3 wave may occur as intermediate product because of the chemical follow-up reaction of LPR (Fig. 1a).

The electrochemical behavior of 4.00 ppm LPR (Ox_1) was investigated between pH 2.00 and 12.00 using DPV and CV techniques at PGE. The peak potential of the oxidation process moved to less positive potentials by raising the pH (until pH 10.00). The plot of the

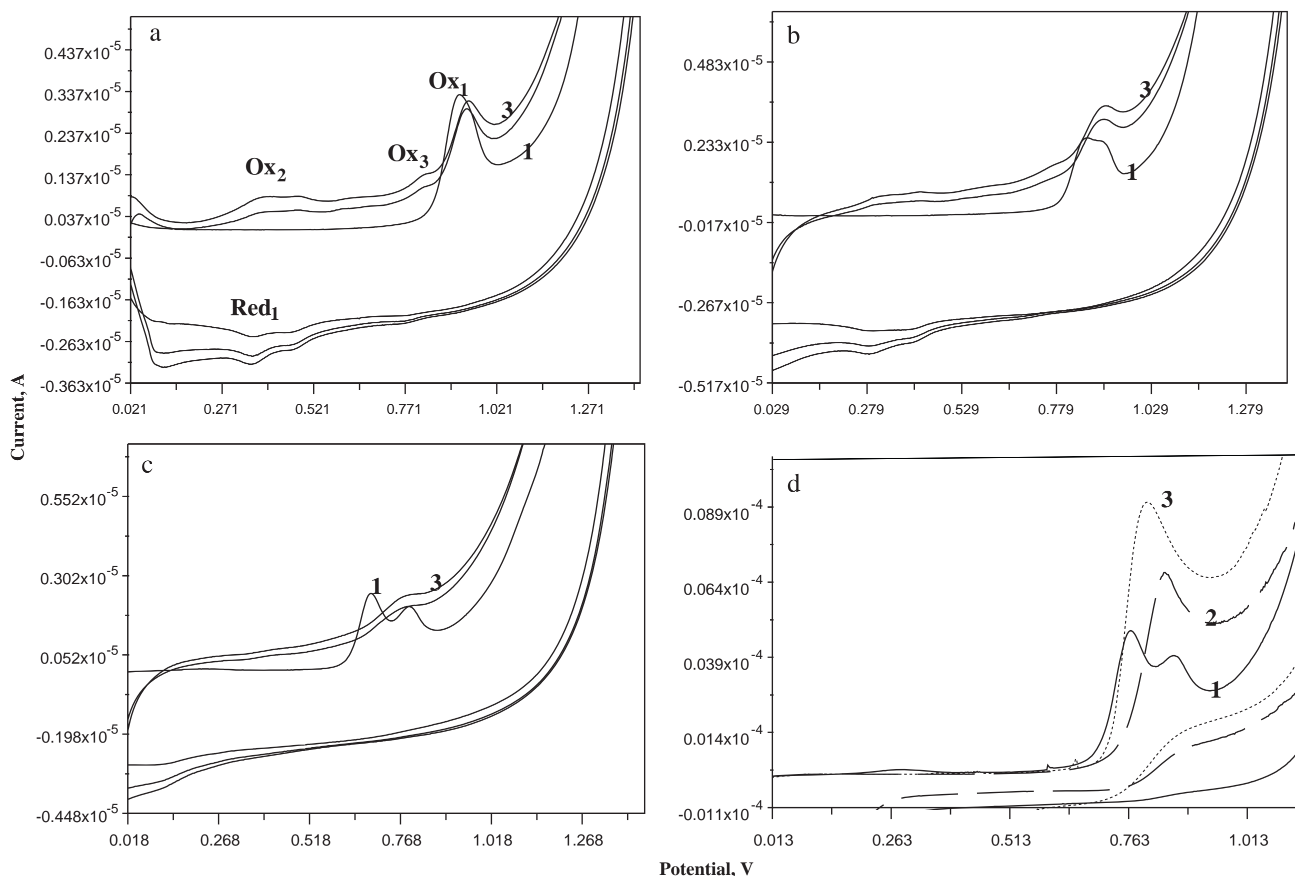

Fig. 1. Multisweep cyclic voltammograms of 4.00 ppm leuprolide solutions in pH 2.00 Britton–Robinson (BR) buffer (a), pH 3.00 BR buffer (b), pH 6.00 BR buffer (c). The numbers indicate the number of scans. (d) is cyclic voltammograms of 16.00 ppm Leuprolide (–––) (1), 8.00 ppm tryptophan (– – –) (2), 8.00 ppm phenol (. . . . . .) (3) solutions in pH 4.80 acetate buffer containing 0.02 M NaCl. Scan rate 100 mV s^{-1}.

peak potential (Ep) versus pH showed one straight line between pH 2.00 and 10.00, which can be expressed by the following equation:

$$\text{Ep}\ \ (\text{mV}) = 1019.5 - 55.32\ \ \text{pH}\ \ \ (r: 0.999)\ \ (n = 9)\ \ \text{for CV}$$

LPR peak potential is almost stable after pH 10.00. The pH independent zone above pH 10.0 means that there are no proton transfer steps before the electron transfer rate-determining step. Since no dissociation occurs before the electron transfer rate-determining step, the oxidation potential remains pH-independent. At pH < pK_a, the conjugate base must be formed by a rapid dissociation of the protonated form. The pH independent point is close to the pK_a value of LPR which is reported in the literature as 9.60 [34]. It can be explained by the changes in protonation of acid–base functions in the molecule. The peak current versus pH experiment shows that the peak current is at its maximum in the acidic media. The experimental results showed that shapes of the curves and the maximum peak current were better in Britton–Robinson buffer at pH 2.0. For this reason, this supporting electrolyte was chosen with respect to their sharp response and better peak shape for the calibration equation.

Scan rate studies, between 5 and 1000 mV s^{-1}, were carried out to assess whether the processes on PGE were under diffusion or adsorption-controlled in 4.00 ppm LPR solutions. The linear increase in the oxidation peak current with the scan rate showed that the adsorption control process is more dominant.

The equation is given below for Britton–Robinson buffer at pH 2.00:

$$\text{ip}\ \ (\mu\text{A}) = 0.019\nu\ \ (\text{mV s}^{-1}) + 0.18\ \ \ r = 0.999\ \ (n: 10)$$

A plot of logarithm of peak current versus the logarithm of scan rate gave a straight line with a slope of 0.85. The obtained slope is close to the theoretical value of 1.0, which is expressed for an ideal reaction of surface species and confirming adsorption-controlled electrode process [35].

The obtained equation is:

$$\log \text{ip}\ \ (\mu\text{A}) = 0.85 \log \nu\ \ (\text{mV s}^{-1}) - 1.33\ \ \ r = 0.997\ \ (n = 10)$$

According to the results obtained from the oxidation peak, the electrochemical reaction was found as adsorption-controlled process. A 102 mV positive shift in peak potential with the absence of the cathodic wave also confirmed the irreversibility of the oxidation process.

Electrochemical results help for the identification of redox mechanism of LPR and provide important information about ds-DNA–LPR interaction points [2,16,17,22–24,39]. LPR is a complex molecule and has two different electroactive moieties that can be oxidized, namely phenol and indole groups [36–38]. To identify the groups responsible for the oxidation of LPR, the drug was compared with some selected model compounds which contain aromatic hydroxyl and/or indole moiety (Scheme 1 and Fig. 1d). Phenol and indole were investigated by cyclic voltammetry at the bare PGE, as a function of pH in order to identify the oxidation process of LPR (not shown). Taking into account all these studies, we suggest that the oxidation processes may be occurring on both the phenol and indole groups together at about the close potential values (Fig. 1d). According to the oxidation peak potentials of the model compounds obtained from the studies performed so far, we may assume that the first oxidation process of LPR is occur-

Table 1
Regression data of the calibration lines for determination of LPR by AdSDPV and guanine for dsDNA–LPR interaction in both media by DPV.

	Bare PGE	On ds-DNA modified PGE	Interaction with ds-DNA in solution phase
Measured potential (V)	0.86	1.02	1.01
Linearity range (ppm)	0.005–0.20	0.20–6.00	0.20–1.00
Number of point	6	5	5
Slope (μA ppm^{-1})	9.950	−0.083	−0.518
Intercept (μA)	0.160	0.736	0.995
SE of slope	4.71×10^{-1}	0.0068	0.0295
SE of intercept	4.43×10^{-2}	0.021	0.0196
Correlation coefficient	0.997	−0.987	−0.995
LOD	1.36×10^{-3}	6.00×10^{-2}	4.00×10^{-2}
LOQ	4.53×10^{-3}	1.80×10^{-1}	1.40×10^{-1}
Within day reproducibility of peak current (RSD%)	1.55	1.75	1.84
Between day reproducibility of peak current (RSD%)	2.03	3.23	2.52

ring on the hydroxyl group of the benzene ring of the molecule (Fig. 1d, curve 3), which is electroactive in both acidic and basic media [39–41]. Ox_2 and Red_1 form a new reversible redox couple can be seen in Fig. 1a and b in acidic media. The results revealed a good agreement with the redox mechanism postulated for model compounds such as anisole and phenol, and suggested that LPR can be determined electrochemically by oxidation of aromatic hydroxyl group. In general, the oxidation of phenol in a solution with high pH will generate the phenoxy radical. At low pH values, the direct oxidation of the phenol, initially forming the radical cation, will become the dominant process [40,41].

It is assumed that a second oxidation step occurs on the nitrogen atom in the indole ring of the molecule, which is electroactive in both acidic and basic media, leading finally to hydroxylation of the benzene ring. A comparative study of indole was performed by cyclic voltammetry as a function of pH to investigate the oxidation of LPR. Taking into account that the cyclic voltammogram of indole is closely match the second peak of LPR in pH 4.80 acetate buffer (Fig. 1d, curve 2). For this reason we may suggest that LPR can be attached on DNA surface by phenol and/or indole part of the molecule (Fig. 1d) which is the electroactive parts of the molecule.

3.2. Analytical parameters and validation of the developed AdSDPV method

Adsorptive stripping pulse voltammetric techniques are effective and rapid electroanalytical techniques with well-established advantages, including good discrimination against background currents and low detection limits [17,19,23,26]. For realizing this study, all necessary adsorptive stripping parameters such as accumulation time and potential were investigated in Britton–Robinson buffer at pH 2.00 on bare PGE. The optimum parameters such as accumulation time and potential were investigated for 1 ppm LPR. 700 mV as accumulation potential and 180 s as accumulation time were selected and applied for further studies. A linear relation in the concentration range between 0.005 and 0.20 ppm was found, indicating that the response was adsorption-controlled in this range. All necessary validation parameters such as LOD, LOQ, within-day and between day reproducibility, specificity, precision, and accuracy were calculated according to the literature [28–30]. The results are listed in Table 1. Freshly prepared and aged (+4 °C, in the dark) LPR solutions were compared for confirming the stability of the solutions. The results demonstrated that the working reference solutions were stable for up to a week period.

3.3. Interaction of LPR with ds-DNA

The electrochemical interaction between LPR and ds-DNA was investigated using two different interaction processes, namely interaction on the PGE surface and interaction in the solution phase. It is known that planar condensed aromatic ring systems play a major role in their interaction with DNA, primarily involving stacking interactions.

3.3.1. Interaction of LPR with ds-DNA on PGE surface

The interaction of LPR with ds-DNA modified PGE was studied by DPV technique. The DPV peak currents of guanine and LPR were measured before and after the interaction. As seen in Fig. 2A, DPV curves were obtained in 0.5 M acetate buffer solutions at pH 4.8 containing 0.02 M NaCl after accumulation of LPR at pre-anodized bare PGE and ds-DNA modified PGE from stirred 1.00 ppm LPR for 90 s at open circuit conditions. The guanine oxidation peak was obtained at about 1.00 V (Fig. 2A; curve 1). The oxidation peaks of LPR occurred at about 0.76 V and 1.17 V (Fig. 2A; curve 3). After

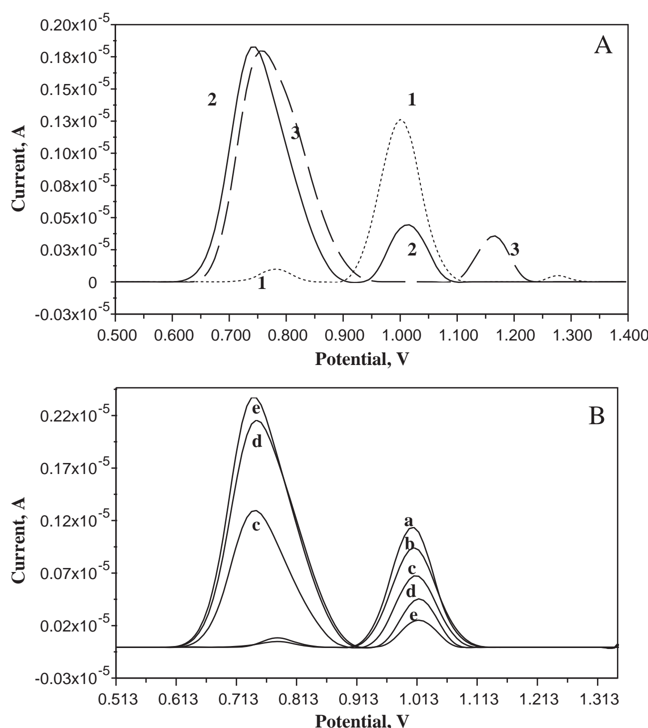

Fig. 2. (A) DP voltammograms for the interaction of 1.00 ppm LPR with 2.00 ppm ds-DNA at PGE surface; guanine oxidation signal before interaction (. . . . . . (1)); after interaction of LPR with dsDNA (––– (2)) and only 1.00 ppm LPR (– – – – (3)) response (at 0.00 V after 90 s accumulation). (B) DP voltammogram for interaction of 2.00 ppm ds-DNA with different amount of LPR solutions at PGE surface: (a) guanine signal obtained from 2.00 ppm ds-DNA solution; (b) after immobilization of ds-DNA on PGE, the obtained guanine signal in acetate buffer solution at pH 4.80 containing 0.02 M NaCl; 2.00 ppm ds-DNA interaction with (c) 0.40 ppm LPR; (d) 2.00 ppm LPR; (e) 6.00 ppm LPR. Experimental conditions as described in Section 2.

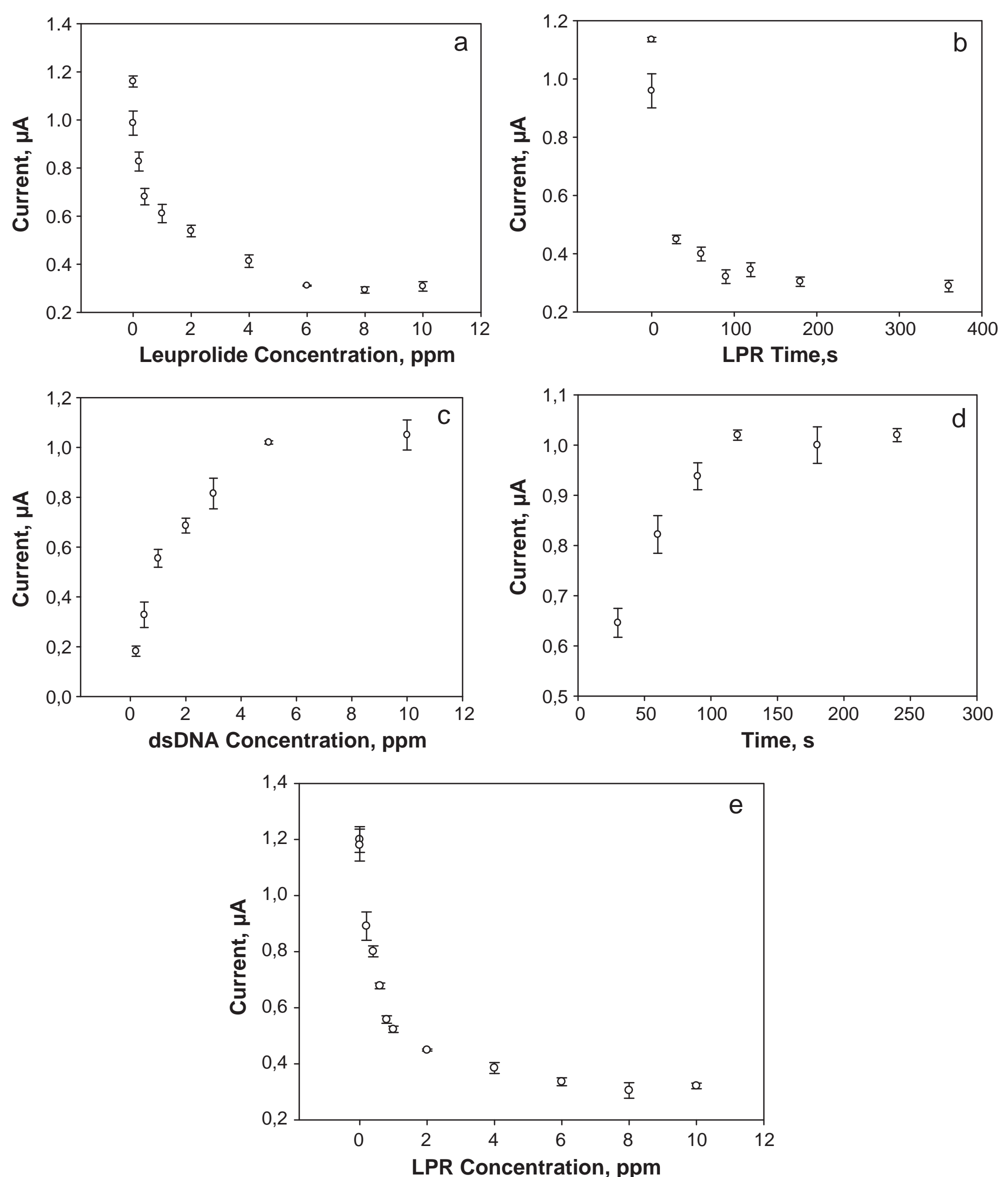

Fig. 3. The effect of LPR at different concentrations (a) and interaction time (b) of LPR with ds-DNA on signal of the guanine at ds-DNA modified PGE, for the optimization of LPR interaction. The effect of ds-DNA concentration (c) and time (d) at oxidation signal at PGE for the optimization of immobilization of ds-DNA in solution phase. The effect of LPR at different concentrations (e) of LPR with ds-DNA on signal of the guanine, for the optimization of LPR interaction in solution phase. The conditions are described in Section 2.

interaction, the oxidation peak potential of the guanine and LPR were obtained at 1.01 V and 0.74 V, respectively (Fig. 2A; curve 2). One of the LPR peaks which were obtained at 1.17 V disappeared after this interaction. The interaction of LPR with ds-DNA modified PGE decreased the oxidation signal of guanine (Fig. 2A; curve 2). After the interaction with LPR, there was a linear decrease at guanine signal (Fig. 2B and 3a). These figures showed that guanine oxidation signal decreased with increasing concentration of LPR up to 6.00 ppm and then it was not changed worth to investigation. After LPR interaction with ds-DNA at PGE surface, the guanine signal of ds-DNA decreased. The decrease of the oxidation signal of guanine bases was attributed to the binding of LPR to this electro active DNA base. This decrease could be explained as a possible damage or shielding of the oxidizable groups of guanine base while LPR interact with ds-DNA either on PGE surface or in solution phase. Hence, it may have caused mutations especially on guanine bases [27,42,43]. These types of experiments are very important to determine DNA sites and rational design of new DNA-targeted molecules for the applications in cancer therapy. Our obtained results showed that PGE might be used for the detection of LPR interaction with ds-DNA directly. Also, this disposable PGE is found suitable and reproducible for the ds-DNA investigation.

The optimum concentration and accumulation time of ds-DNA was studied for obtaining the precision and reproducibility of the guanine signal as given in our previous paper [26]. For finding the optimum concentration of ds-DNA, seven different concentrations between 0.2 and 10 ppm were studied. These studies were realized at 0.5 V using 120 s accumulation times [26]. The best results were obtained with 2 ppm ds-DNA concentration (figure not shown) [26].

The effects of the experimental parameters; concentration of LPR, accumulation time of ds-DNA and LPR were also studied to find optimum analytical conditions. For obtaining the relationship style between LPR and ds-DNA, different LPR concentrations were studied between 0.00 and 10.00 ppm concentration levels. These studies were realized using 0 V accumulation potential at optimum

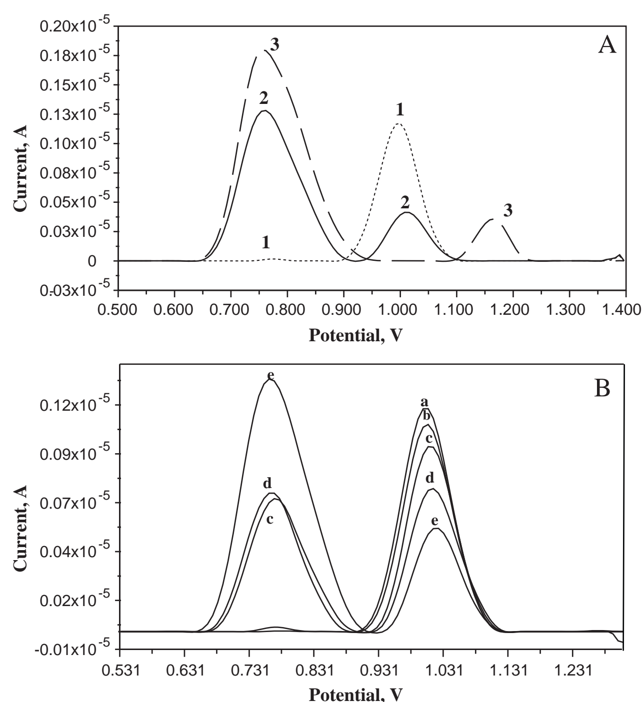

Fig. 4. (A) DP voltammograms for the interaction of 1.00 ppm LPR with 5.00 ppm ds-DNA in solution phase; guanine oxidation signal before interaction (...... (1)); after interaction of LPR with dsDNA (––– (2)) and only 1 ppm LPR (– – – – (3)) response (at 0.00 V after 120 s accumulation). (B) DP voltammogram for interaction in solution phase of 5.00 ppm ds-DNA with different concentrations of LPR: (a) guanine signal obtained from 5.00 ppm ds-DNA solution; (b) after ds-DNA on PGE, the obtained guanine signal in acetate buffer solution at pH 4.80 containing 0.02 M NaCl; 5.00 ppm ds-DNA interaction with (c) 0.20 ppm LPR; (d) 0.40 ppm LPR; (e) 1.00 ppm LPR. Experimental conditions as described in Section 2.

accumulation time (60 s). Other parameters were described in Section 2. The best result was obtained at 6.00 ppm LPR concentration (Fig. 3a). After this step, using 2.00 ppm ds-DNA and 6.00 ppm LPR concentrations, the optimum accumulation time studies were realized between 0 and 360 s. The optimum accumulation time was obtained as 90 s (Fig. 3b).

The within day reproducibility results (RSD%) for guanine potential and current were 0.35% and 1.01%, respectively. Between day reproducibility results for peak potential and peak current were 0.43% and 1.06%, respectively. These results were obtained from 5 individual experiments.

3.3.2. Interaction in solution phase between ds-DNA and LPR

For the confirmation of the interaction between ds-DNA and LPR, the following study was also realized. For finding the optimum concentration of ds-DNA in solution, 7 different concentrations between 0.20 and 10.00 ppm were studied. These studies were realized at +0.00 V using 120 s accumulation time. Other parameters are the same as described in Section 2. The best results were obtained with 5 ppm ds-DNA solutions as shown in Fig. 3c. After this step, the accumulation times were realized between 30 and 240 s using 5.00 ppm ds-DNA concentrations. Optimum accumulation time was obtained using 120 s as shown in Fig. 3d. After the interaction with LPR, there was a linear decrease obtained at guanine signal using interaction in solution techniques (Fig. 3e).

Fig. 4A shows typical DPV of 5.00 ppm ds-DNA solution with and without adding LPR at PGE in acetate buffer (0.02 M, pH 4.80). The potential value of the guanine oxidation signal was at about 1.00 V (Fig. 4A, curve 1). The oxidation peaks of LPR occurs at about +0.75 V and +1.17 V, alone (Fig. 4A, curve 3). After interaction, the oxidation peak potential of the guanine and LPR were obtained at +1.01 V and +0.76 V, respectively (Fig. 4A, curve 2). In addition to the decrease in guanine signal, the second oxidation peak of LPR was disappeared (Fig. 4A, curve 2) at more positive potential (at about + 1.17 V), after interaction of LPR with ds-DNA. After the interaction with LPR, there was a linear decrease at guanine signal (Fig. 4B).

DPV curves were recorded after addition of different amounts of LPR to the solutions containing 5.00 ppm ds-DNA. The changes in the electrochemical signals obtained from LPR-ds-DNA complex were compared with the signals of pure DNA in the solution. The peak currents corresponding to the oxidation signal of guanine from ds-DNA interaction in solution phase studies were decreased after addition of each LPR amount between 0.20 and 10.00 ppm.

The within day reproducibility results (RSD%) for guanine peak potentials and currents were 0.35% and 1.39%, respectively. Between day reproducibility results for peak potentials and peak currents were 0.41% and 2.39%, respectively. These results were obtained from 5 individual experiments.

3.4. Electroanalytical determination of LPR using ds-DNA modified PGE and interaction in solution phase techniques

The results obtained for the LPR oxidation using ds-DNA biosensor demonstrates a good possibility for developing an electroanalytical methodology for LPR detection and determination. A linear dependence between analytical signals and LPR concentrations which considered as the decreasing in the guanine signal after interaction with LPR was observed a linear region for both interaction techniques. The electroanalytical behavior of LPR is dependent on the electrolyte solutions using both interaction techniques with ds-DNA. For this electroanalytical study, 0.50 M acetate buffer at pH 4.80 containing 0.02 M NaCl was used as the supporting electrolyte. In this supporting electrolyte solution, the best peak shape and separation from the background currents were obtained. The decreasing in the guanine oxidation peak currents were used as a function of LPR concentrations with ds-DNA modified PGE and interaction in solution phase with ds-DNA to confirm the linear relationship between the current and concentration (Figs. 2B and 4B). In the proposed ds-DNA modified PGE method and interaction in solution phase method, the peak current is decreased linearly between 0.20 and 6.00 ppm and between 0.20 and 1.0 ppm concentration range, respectively. The characteristics and necessary validation parameters of these calibration plots are summarized in Table 1. Both the LOD and LOQ values confirmed the sensitivity of the proposed method (Table 1) which is calculated using following equations:

$$\mathrm{LOD} = \frac{3s}{m}; \qquad \mathrm{LOQ} = \frac{10s}{m},$$

where s is the standard deviation of the peak current (three runs) of the lowest concentration of the linearity range, m is the slope of the related calibration curve [28–30,44]. The precision of the methods was evaluated by repeating experiments on the same day (within day reproducibility) in different standard solutions (freshly prepared but at the same concentration) and over a week period (between days reproducibility) from different standard solutions. The within day and between day reproducibility results were calculated after interaction with ds-DNA for both techniques were reported in Table 1.

On the basis of the obtained results, both on ds-DNA modified PGE and interaction with ds-DNA in solution phase were applied to the direct determination of LPR in intravenous injection dosage forms, using related calibration straight lines.

Table 2
Results obtained for LPR determination in pharmaceutical dosage form using bare PGE and dsDNA modified PGE and in solution phase methods.

	Bare PGE	On dsDNA modified PGE	Interaction with ds-DNA in solution phase
Labeled claim (mg/mL)	5.00	5.00	5.00
Amount found[a] (mg/mL)	4.97	5.04	4.98
RSD%	1.89	3.15	2.34
Bias%	0.60	−0.80	0.40
$t_{\text{calculated}}$	$t_{\text{theoretical}}$: 2.31	0.46	0.91
$F_{\text{calculated}}$	$F_{\text{theoretical}}$: 2.60	0.33	0.69

[a] Each value is the mean of five experiments.

3.5. Determination of LPR in injection solution using ds-DNA modified PGE and interaction in solution phase techniques and also with bare PGE

Pretreatment was not required for the samples preparation such as time-consuming extraction, evaporation or filtration steps prior to the analysis. On the basis of the above results, all proposed methods for the assay of LPR, namely ds-DNA modified PGE, interaction with ds-DNA in solution phase method and oxidative assay on bare PGE were applied to the direct determination of LPR in intravenous injection dosage forms (Table 2). There is no official or reference method reported in any pharmacopoeias or in literature so far for the determination of LPR in its dosage form. For this reason, the proposed methods were compared with each others. The *F*- and Student *t*-tests were carried out on the data and statistically examined the validity of the obtained results using DPV at ds-DNA modified PGE and interaction with ds-DNA in solution phase and bare PGE methods. At the 95% confidence level, the values of *t*- and *F*-tests (calculated from the experiments) were less than that of theoretical *t*- and *F*-values showing that there are no significant differences between the proposed methods. The proposed methods can successfully be applied for LPR assay in injection dosage form without any interference. The accuracy of the analysis of all methods was determined by calculating the relative error (Bias%) between the measured mean concentrations and actual concentration. The precision value around the mean value should not exceed 5% of the RSD% [28–30,44] (Table 2).

4. Conclusion

The investigations of drug–DNA interaction would provide new compounds to be tested for an effect on a biochemical target, for the design of DNA biosensors, which will further become DNA microchip systems [3]. At the present work, sensitive and selective DNA biosensor was developed for the determination of LPR in vitro. DNA biosensors eliminate the need for some difficult analyze technique [22,23]. The utility of this electrochemical biosensor for interaction between ds-DNA and LPR is cost effective and it provides rapid detection. The proposed electroanalytical method is experimentally convenient, sensitive, selective and rapid so that it requires only small amounts of materials. The ds-DNA modified PGE was used in combination with DPV to obtain the information about the interaction of LPR with ds-DNA, based on the changes at guanine signal. The changing of guanine signal that obtained using interaction with ds-DNA in solution phase is also confirmed this interaction. The electrochemical DNA biosensor technique has produced experimental evidence for the LPR–ds-DNA interaction and these results contribute to the understanding of the anticarsinogenic activity of the important anticancer drugs.

As a result of the interaction of LPR in different concentrations with ds-DNA, a decreasing was observed in the response based on the signal of guanine. This phenomenon could be explained by damage on the oxidizable groups of electroactive base guanine because of the adsorption of LPR [45]. This biosensor was also used for the determination of LPR for the first time. Short pre-concentration time permits convenient measurements of low concentrations of LPR. The results have also shown that these studies can play a key role in developing newly produced chemotherapeutic compounds. Also the usage of these voltammetric methods for drug–ds-DNA interactions will enable the discovery of unknown drug–ds-DNA interaction mechanisms.

The proposed ds-DNA modified PGE or interaction in solution methods were not only used for the detection of LPR but also used for the determination of this compound in raw material and pharmaceutical dosage form. Also bare PGE was successfully applied for the assay of LPR in pharmaceutical dosage forms. The advantages of the proposed methods are the extremely low detection limits and suitability for online and in situ measurements. Further developments in the electrochemical biosensors will provide a screening of a large number of new drug compounds for their anticancer activities which may lead to the design of effective drugs with fewer side effects.

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