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Different interaction of codeine and morphine with DNA: A concept for simultaneous determination

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ABSTRACT

In this study, the interaction between codeine and morphine with dsDNA was assessed at pH 7.0. Poly(diallyldimethylammonium chloride), PDDA, was used as a dispersant of MWCNTs. Using differential pulse voltammetry (DPV) at pencil graphite electrode (PGE) showed that both molecules were electrochemically oxidized due to the presence of phenolic and amino groups in their structures. When DNA was added to the solution, the electrochemical signal of codeine and morphine was decreased and shifted to more negative and positive potentials, respectively. The interaction modes were respectively electrostatic for codeine and intercalation for morphine with two anodic peaks of codeine being merged into them when DNA concentration was increased. At high DNA concentrations, a sharp anodic wave for codeine and a clear discrimination of codeine and morphine oxidation peaks were observed. Finally, a pencil graphite electrode was modified with carbon nanotubes and DNA was tested in order to determine codeine and morphine in solution. Electrochemical oxidation of codeine and morphine bonded on dsDNA/MWCNTs–PDDA/PGE was used to obtain an analytical signal. Allowing five min as an accumulation time, a linear dependence was observed between 0.05 and 40 $\mu g\,mL^{-1}$ for codeine and 0.05 and 42 $\mu g\,mL^{-1}$ for morphine. Detection limits of 0.041 and 0.043 $\mu g\,mL^{-1}$ were obtained for codeine and morphine, respectively. The biosensor was applied to validate its capability for the analysis of codeine and morphine in blood serum, urine samples and pharmaceutical formulations.

1. Introduction

Codeine and morphine, the main alkaloids in poppy seeds, have pharmacological and toxicological activity. They are widely used as narcotic analgesic and antitussive drugs (Raffa, 1996). Following codeine administration, morphine is only present in low concentrations in plasma and urine but contributes substantially to codeine's analgesic effect. Simultaneous determination of codeine and morphine in biological samples is a common practice in many laboratories involved in forensic and clinical toxicology. In addition, it is pharmacologically important to be able to determine codeine and its metabolite in biological samples.

So far, several methods have been reported for the detection of them. Spectrophotometry (Elsayed et al., 1979), spectrofluorimetry (Ramos Martos et al., 2001; Zhang et al., 2000), chemiluminescence (Greenway et al., 2000; Idris and Alnajjar, 2008), high performance liquid chromatography (He et al., 1998; Berga et al., 2009) and gas chromatography (Meatherall, 1999; Hofmann et al., 1999) have been used for this purpose. Many of these methods require several manipulation steps such as liquid–liquid extraction that are time consuming and solvent-usage intensive and need sophisticated instrumentation and training. Electroanalytical methods have attracted more attention in recent years for pharmaceutical determination due to their sensitivity, accuracy, lower cost, and simplicity. The use of voltammetric methods with modified electrodes was recently recommended for the determination of morphine in real samples. Clay modified screen-printed carbon electrode (Shih et al., 2002), Nafion/ruthenium oxide pyrochloride chemically modified electrode (Zen et al., 1998), palladium modified aluminum electrode (Pournaghi-Azar and Saadatirad, 2010), cobalt hexacyanoferrate modified glassy carbon electrode (Xu et al., 2002) and multiwall carbon nanotubes (MWCNTs) modified pretreated glassy carbon electrode (Salimi et al., 2005) have been used for the determination of codeine or morphine.

Molecules of codeine and morphine are structurally similar and usually interfere with each other during simultaneous analysis in some real samples. To date, most of the analytical methods reported for the quantification of morphine and codeine in biological samples have involved the application of GC–MS (Brewer et al., 2001; Watson et al., 1995), liquid chromatography (Bogusz et al., 1997; Coles et al., 2007), electrophoresis with UV

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detection (Ahsanul and Stewart, 1999) and electrochemical (Zhou et al., 2002) detection. These methods, being complicated, costly and time consuming, require hydrolysis and derivatization for GC–MS analysis and sample preparation involves for liquid chromatography analysis.

Voltammetric and amperometric determination of morphine and codeine, using Prussian blue film modified-palladized aluminum electrode, has been reported by Pournaghi-Azar and Saadatirad (2008). Prussian blue film on the non-palladized aluminum (naked aluminum) is not electroactive and any oxidation peaks relevant to morphine and codeine oxidation did not appear on the unmodified palladized aluminum electrode. The major advantages of palladized aluminum electrode are the simplicity, fast response (few min) and high stability of the modifier on the electrode surface. The linearity ranges of codeine and morphine are 2–30 and 2–50 μmol L^{-1}, respectively, with the detection limits of 0.8 μmol L^{-1}. These researchers did not study the influence of potentially interfering substances and did not applied the sensor for analysis of codeine and morphine in real samples such as pharmaceutical formulations, blood serum and urine.

The electroactivity of nucleic acids has allowed the development of more sensitive and rapid electrochemical techniques, based on the differences in the electrochemical behavior of DNA-targeted molecules and DNA (Ensafi et al., 2012a, 2012b; Fojta et al., 2008). Electrochemical DNA biosensors are fabricated by immobilizing DNA on the electrode surface (Ensafi et al., 2012a, 2012b). Binding of small molecules to DNA on the electrode surface alters the electrochemical properties of DNA and/or target molecule. This change could be a signal for the determination of molecules interacting with DNA. This paper reports the development of a DNA functionalized biosensor with an electroanalytical methodology appropriate for routine analysis of morphine and codeine. In this study, a DNA based biosensor was constructed through layer-by-layer technique. First, poly(diallyldimethylammonium chloride), PDDA, was used as a dispersant of MWCNTs. Then, MWCNTs–PDDA was immobilized on the surface of electrochemically pretreated pencil graphite electrode (PGE) to increase the electron transfer characteristics of the electrode surface. Finally, the dsDNA polyions were immobilized at the surface of MWCNTs–PDDA/PGE. The DNA-modified PGE was used as an analytical tool to evaluate the interaction of codeine and morphine with the dsDNA. The new biosensor was used for the determination of codeine and morphine in blood serum, urine samples and pharmaceutical formulations.

2. Experimental

2.1. Chemicals

All chemicals were of reagent grade and used as received without further purification. Codeine phosphate and morphine sulfate were purchased from Temad Co., Iran. Codeine and morphine working solutions for voltammetric investigations were prepared by the dilution of the stock with 0.1 mol L^{-1} phosphate buffer solutions at pH 7.0, containing 0.02 mol L^{-1} NaCl.

Reagent grade Tris–HCl, MWCNTs, H_3PO_4, NaCl and NaOH were purchased from Aldrich Chemicals (Milwaukee, USA). Double stranded salmon sperm dsDNA and PDDA (low molecular weight) were purchased from Sigma (St. Louis, USA). The dsDNA was dissolved in Tris buffers and the stock solution was stored at 4 °C.

MWCNTs used in this work were from Fluka and have a diameter of 7–11 nm and a length of 5–9 μm.

2.2. Apparatus

Electrochemical and impedimetric measurements were performed using an Autolab PGSTAT 12, potentiostat/galvanostat connected to a three-electrode cell, Metrohm Model 663 VA stand, using a GPES 4.9 software package (Eco Chemie, The Netherlands). An Ag/AgCl/3 mol L^{-1} KCl and a platinum wire were used as the reference and counter electrodes, respectively. A standard one-compartment three-electrode cell of 10 mL capacity was used in all experiments. A renewable pencil graphite electrode (PGE), as described by Erdem et al. (2003), was used in all experiments. A Noki pencil was used as a holder for Pentel graphite leads. Electrical contact with the lead was obtained by soldering a metallic wire to the metallic part. The pencil was held vertically with 12 mm of the lead being extruded outside (9 mm of which was immersed in the solution). All electroanalytical measurements were performed at room temperature.

Metrohm pH meter (Model 827) with a glass electrode (Corning) was used to adjust the solution pH.

2.3. Functionalization and purification of MWCNTs

MWCNTs were purified and functionalized, as described elsewhere (Ghica et al., 2009). A mass of 120 mg of MWCNTs was stirred in 10 mL of a 3 mol L^{-1} nitric acid solution for 20 h. The solid product was collected on a filter paper and washed several times with pure water until the filtrate solution was neutral (pH 7.0). The obtained functionalized MWCNTs were then dried in an oven at 80 °C for 24 h.

2.4. Preparation of the DNA biosensor

The surface of the PGE was pretreated by applying +1.40 V for 60 s in 0.5 mol L^{-1} acetate buffer (pH 4.8). An aqueous solution of 1.0 mg mL^{-1} PDDA was initially prepared with 0.5 mol L^{-1} NaCl. Then, 1.0 mg MWCNT was dispersed into 1.0 mL PDDA solution. The mixture was sonicated for 3 h to obtain a homogeneous black suspension, which was sonicated for 15 min immediately before preparing the PDDA–MWCNTs films. PGEs were dipped in these composites for 30 min. The electrodes were then dipped in 0.1 mol L^{-1} NaOH solution for 5 min, before being placed in the deionized water. The PDDA–MWCNTs/PGE was dried in N_2 stream. The positively charged PDDA–MWCNTs/PGE was subsequently dipped into DNA solution (1.0 mg mL^{-1} buffered with Tris–HCl, pH 7.0) for approximately 10 min, along with water rinsing and N_2 drying. The modified PGEs thus obtained were designated as dsDNA/PDDA–MWCNTs/PGE in this study.

2.5. Cyclic voltammetry and electrochemical impedance spectroscopy of $K_3[Fe(CN)_6]$

The cyclic voltammogram of 5.0 mmol L^{-1} $K_3[Fe(CN)_6]$/$K_4[Fe(CN)_6]$ in 0.1 mol L^{-1} KCl was recorded from −0.20 to 0.60 V, using a scan rate of 100 mV s^{-1}. Electrochemical impedance spectroscopy was done at a polarization potential of 0.15 V in a frequency range of 0.005–10^5 Hz and at amplitude of 10 mV.

2.6. Adsorptive stripping differential pulse voltammetry (ASDPV) at dsDNA/PDDA–MWCNTs/PGE

The dsDNA/PDDA–MWCNTs/PGE was initially immersed into a well-stirred sample solution (400 rpm) containing the desired codeine and/or morphine concentration in a phosphate buffer solution (0.1 mol L^{-1}, pH 7.0), PBS, for a given time under open circuit conditions and then rinsed with the PBS. Finally, voltammetric stripping step was performed with a positive-going

differential pulse potential scan (from 0.0 to 0.90 V), using a pulse amplitude of 50 mV, a modulation time of 0.05 s, and a step potential of 8 mV in the blank PBS (0.1 mol L^{-1}, pH 7.0). The raw data was treated using the Savitzky and Golay filter (level 2) of the GPES software, followed by the GPES software moving average baseline correction, using a "peak width" of 0.01.

2.7. Preparation of real samples

One milliliter of morphine sulfate injection (APP Pharmaceuticals, LLC Schaumburg 0.5 mg mL^{-1}) was directly transferred into a 100-mL volumetric flask and diluted to the mark using 0.1 mol L^{-1} phosphate buffer (pH 7.0). Each time, 10.0 mL of this solution and a proper volume of the standard solution (including codeine or morphine) were transferred into the electrochemical cell.

For codeine analysis, 0.5 mL of expectorant codeine syrup was transferred into a 100-mL volumetric flask and diluted with 0.1 mol L^{-1} phosphate buffer (pH 7.0) to the calibration mark. The resulting stock solution was diluted with supporting electrolyte (dilution factor 1/1500). The solution was transferred into the voltammetric cell and the DP voltammograms were recorded in a way similar to the pure drug. Standard addition method was used to evaluate the content of codeine in the tablet.

Urine samples were stored in a refrigerator (at 4 °C) immediately after collection. 10 mm of the sample was centrifuged for 10 min at 2000 rpm. The supernatant was filtered using a 0.45 μm filter and then diluted four times with the phosphate buffer pH 7.0. The solution was transferred into the voltammetric cell to be analyzed without any further pretreatment. Standard addition method was used for the determination of morphine and codeine in the samples.

In order to precipitate proteins in the plasma samples, 1.0 mL of the samples was treated with 20 μL perchloric acid ($HClO_4$, 20% v/v). Then, the mixture was vortexed for a further 30 s and then centrifuged at 6000 rpm for 5 min. The solution was diluted five times with water and transferred into the voltammetric cell to be analyzed without any further pretreatment. Standard addition method was used for the determination of morphine and codeine in the samples.

GC–MS analysis is described in the supplementary materials.

3. Results and discussions

3.1. Characteristics of dsDNA/PDDA–MWCNTs/PGE

3.1.1. AFM and SEM characterization

Fig. 1 shows typical AFM and SEM images of the bare PGE (Figs. 1A and B, respectively), the PG substrate modified with MWCNTs–PDDA (Figs. 1C and D, respectively) and dsDNA/PDDA–MWCNTs/PGE (Figs. 1E and F, respectively). The surface roughness of unmodified PGE is clearly seen in Figs. 1A and B. The graphite layers can be seen well in Fig. 1B. Cationic polyelectrolyte PDDA can be wrapped on the sidewall surface of the carboxylated MWCNTs for loading negative charge of the dsDNA by a layer-by-layer assembly. In this work, the positively charged PDDA–modified carboxylated MWCNTs were employed for the electrostatic adsorption of the negatively charged dsDNA. The AFM and SEM images of PDDA–MWCNT film showed a homogeneous surface and good dispersion (Figs. 1C and D). When MWCNTs were sonicated and incubated in PDDA solution, the positive charged polyelectrolyte PDDA could be adsorbed (or warpped) on the sidewall surface of the MWCNTs, resulting in positively charged PDDA–modified MWCNTs. After MWCNTs–PDDA modification onto the PGE, the value of the diameter for MWCNTs is significantly increased. This confirms that PDDA has been adsorbed on MWCNTs. This uniform and interdigitated structure provided a significant increase of effective area for the dsDNA loading. The negative charge of the DNA made it be suitably incorporated in positively charged PDDA–MWCNTs, forming DNA/MWCNTs–PDDA/PGE nanocomposite. The AFM and SEM images indicate the obvious morphological change between PDDA–MWCNTs and dsDNA loaded PDDA–MWCNTs (Fig. 1E). After loading dsDNA, the clear MWCNTs images became dim (Fig. 1F). This demonstrates that PDDA can be used as a good matrix for MWCNTs and DNA. PDDA acts as a polymer backbone to give a stable and homogeneous thin film.

3.1.2. Cyclic voltammetry of $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$

Fig. 2A shows the cyclic voltammograms of 5.0 mmol L^{-1} $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ containing 0.1 mol L^{-1} KCl at the surface of different electrodes. As can be seen in Fig. 2A, bare PGE has a pair of well-defined voltammetric peaks with a cathodic peak potential (E_{pc}) of 0.420 V and an anodic peak potential (E_{pa}) of 0.103 V. The peak-to-peak separation (ΔE_p) is 317 mV (Fig. 2A, curve a). The peak current was increased dramatically after coating the PGE with the

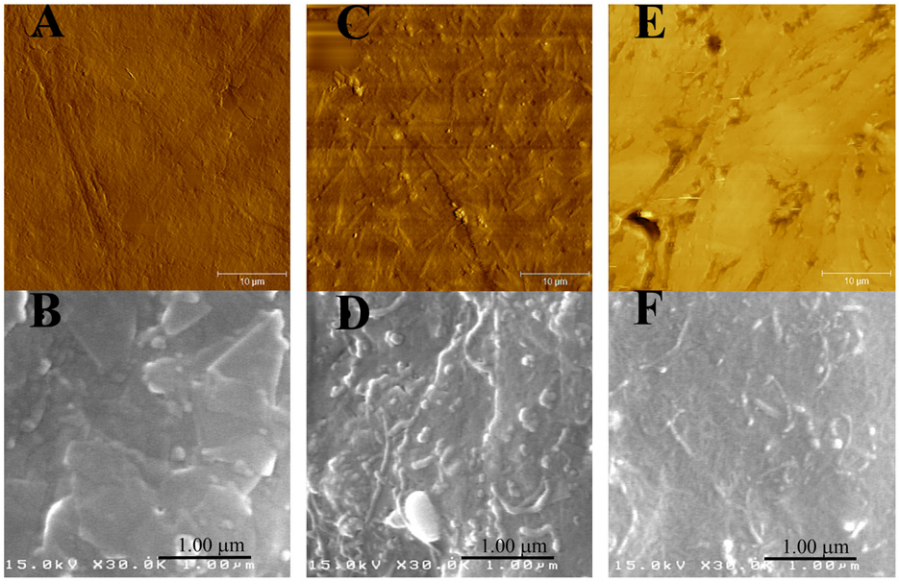

Fig. 1. AFM and SEM images of unmodified PGE (A and B), MWCNTs–PDDA modified PGE (C and D), and DNA/MWCNTs–PDDA modified PGE (E and F), respectively.

MWCNTs–PDDA nanocomposite and its ΔE_p was decreased to 160 mV (Fig. 2A, curve b). The strong improvement of the redox probe cyclic voltammogram shape was found at MWCNTs–PDDA/PGE. This should be attributed to the homogenous distribution of MWCNTs with high electrical conductivity on the electrode surface using the PDDA matrix. Therefore, MWCNTs–PDDA nanocomposite modified electrode can promote electron transfer reactions better than the bare PGE. When the dsDNA was immobilized on the MWCNTs–PDDA/PGE, the $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ current signal was decreased (Fig. 2A, curve c). At the same time, an increase in the ΔE_p to 324 mV can be observed and evaluated due to a decrease of the electrochemical reversibility of the $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ redox couple at the DNA modified electrode. A possible reason could be that the negatively charged dsDNA hindered the $[Fe(CN)_6]^{3-/4-}$ from participating in the electrode reaction on the surface. This phenomenon also demonstrated that the dsDNA was successfully immobilized on the modified PGE surface.

3.1.3. Electrochemical impedance spectroscopy

Fig. 2B presents the Nyquist plots of a bare PGE, MWCNTs–PDDA/PGE and dsDNA/MWCNTs–PDDA/PGE in 5.0 mmol L^{-1} $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) containing 0.1 mol L^{-1} KCl. It was found that the impedance of the electrode was drastically decreased in the presence of MWCNTs–PDDA nanocomposite. This is due to the fact that the nanocomposite of MWCNTs and PDDA promoted the electron exchange between $[Fe(CN)_6]^{3-/4-}$ in the solution and the electrode surface. Moreover, an obvious increase in the interface impedance was observed when the dsDNA was immobilized on the surface of MWCNTs–PDDA/PGE. The higher R_{ct} is the direct experimental evidence for the successful adsorption of the dsDNA on the surface of MWCNTs–PDDA/PGE. It is suggested that the dsDNA could slow down the rate of electron transfer due to electrostatic repulsion between negatively charged dsDNA and $[Fe(CN)_6]^{3-/4-}$. The impedance results are in good agreement with the cyclic voltammograms obtained under similar conditions.

3.2. Interaction between codeine and morphine with DNA

3.2.1. Interaction of codeine and morphine with the DNA in solution

Codeine is a complex molecule that can be oxidized with different groups. Anodic oxidation of codeine follows a rather complex mechanism that is pH dependent. Typical DPV behaviors of 30.0 μg mL^{-1} codeine at different pHs (PBS, 0.1 mol L^{-1}) are shown in Fig. S-1. From pH 2.0 to 8.0, two anodic waves were observed (Fig. S-1a). These peaks might be related to the oxidation of the tertiary amine and the 6-hydroxy groups of codeine (Garrido et al., 2004a). At pH 8.0, three anodic peaks for codeine could be observed (Fig. S-1b). The appearance of a new wave can be attributed to the oxidation of the 3-methoxy group. At pH 9.0 and 10.0, four anodic waves were observed (Fig. S-1c). The new peak is also probably related to a subsequent oxidative process involving the secondary amine group from the oxidation of the tertiary amine group present in codeine (Garrido et al., 2004a). Typical DPV behavior of 30.0 μg mL^{-1} codeine in the absence and presence of different concentrations of the dsDNA at pH 7.0 are shown in Fig. 3A. The anodic peaks potential (E_{pa}) in the absence of the dsDNA are located at 0.90 and 1.00 V. As indicated by the DPV in Fig. 3A, the equilibrium concentration of codeine in the presence of the dsDNA declines, leading to a decrease in the peak current (i_p) with a negative shift of the anodic peak potential (E_{pa}) by 50 mV in the presence of 20.0 μg mL^{-1} DNA. Moreover, E_{pa} shifts to more negative potentials and i_p obviously continues to decrease with increasing the dsDNA concentration (Fig. 3A). Under the experimental conditions of Fig. 3, pure dsDNA displays no electrochemical signals in this potential range at the PGE. This is mainly due to the merging of the anodic peak at low dsDNA concentrations and the background discharge (Ibrahim, 2001). According to these observations, it seems that decreases in the peak currents of codeine after the addition of the dsDNA are caused by the binding of codeine with the bulky, slowly diffusing the dsDNA, resulting in a considerable decrease in the apparent diffusion coefficient (D). According to the literature (Carter et al., 1980), the negatively shifted peak potential is characteristic of an electrostatic mode. If the peak potential shifts positively, the interaction will be an intercalative mode. It may be claimed that codeine is bound to DNA mainly through an electrostatic mode. Furthermore, two anodic peaks of codeine were merged and incorporated at high concentrations of the dsDNA. Since these two anodic waves might be related to the oxidation of the tertiary amine and the 6-hydroxyl groups, different interactions of these groups with the dsDNA are expected to give rise to different shifts in their peak potentials and to their different merging patterns in the presence of the dsDNA.

Morphine was electrochemically oxidized with one anodic peak observed in the potential range under study at pH 7.0 (Fig. 3B). This peak might be related to the oxidation of the phenolic group (Garrido et al., 2004b). As shown in Fig. 3B, the oxidation signal of morphine is decreased with increasing the dsDNA concentration.

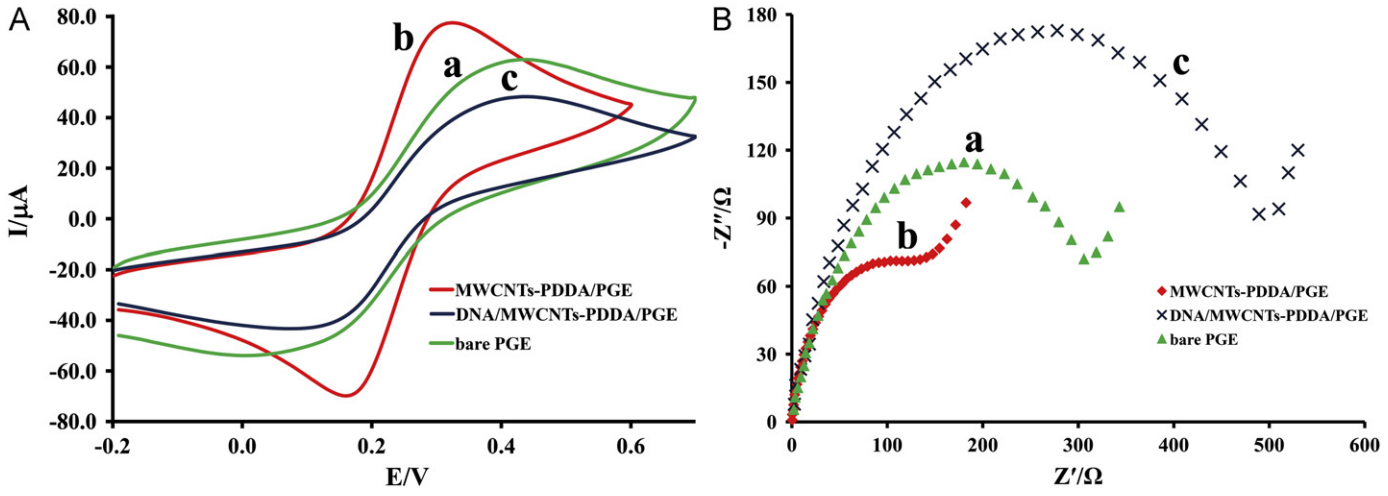

Fig. 2. CVs (A) and impedance spectra (B) of (a): bare PGE (b): MWCNTs–PDDA/PGE; and (c): DNA/MWCNTs–PDDA/PGE in 5.0 mmol L^{-1} $Fe(CN)_6^{3-/4-}$ containing 0.1 mol L^{-1} KCl. Conditions: CV measurement at 100 mV s^{-1} scan rate between +0.20 and +0.70 V.

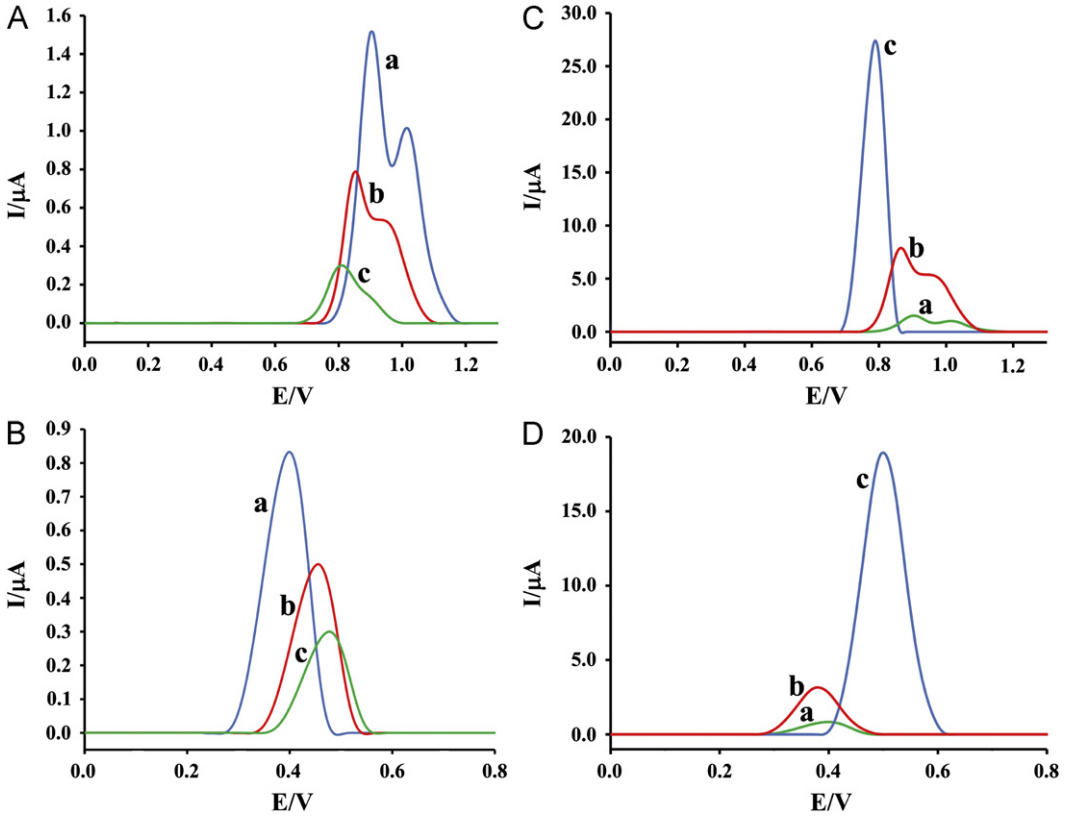

Fig. 3. DPVs of the bare PGE in: (A) 30.0 μg mL^{-1} codeine, and (B) 30.0 μg mL^{-1} morphine containing (a) 0 μg mL^{-1}; (b) 20.0 μg mL^{-1}; and (c) 30.0 μg mL^{-1} of the dsDNA; DPVs of 30.0 μg mL^{-1} codeine (E) and 30.0 μg mL^{-1} morphine (F): (a) at the bare PGE; (b) at MWCNTs–PDDA/PGE; and (c) at dsDNA/MWCNTs–PDDA/PGE with an accumulation time of 300 s. Conditions: 0.01 mol L^{-1} phosphate buffer (pH 7.0).

Additionally, a significant positive change was observed in the peak potential after interaction with the dsDNA. The positive shift of the peak potential could allow us to suggest an intercalation mode for the interaction of morphine with DNA.

3.2.2. *Interaction of codeine and morphine with the DNA at dsDNA/MWCNTs–PDDA/PGE*

DPVs of 30.0 μg mL^{-1} codeine (Fig. 3C) and morphine (Fig. 3D) at the bare PGE, MWCNTs–PDDA/PGE and dsDNA/MWCNTs–PDDA/PGE are displayed. These studies were performed in open circuit conditions using 300 s accumulation time. As can be seen, when the PGE surface was treated with the dsDNA, an increasing in i_p over 18-time and 23-time was observed for 30.0 μmol L^{-1} codeine and morphine, respectively. In addition, E_p was shifted negatively by 112 mV for codeine and positively by 100 mV for morphine. Furthermore, two anodic peaks of codeine were merged and incorporated with increasing the dsDNA concentration. The results showed that the dsDNA-modified surface was more favorable for codeine and morphine molecules to undergo redox reaction than the bare PGE. It was an indication of a preconcentration of codeine and morphine on the DNA-modified surface due to binding to the dsDNA. The sharp anodic peak also shows that codeine and morphine underwent redox through the intercalated state. As the linear plot of i_p versus the scan rate (Fig. S-2) revealed, codeine and morphine are strongly adsorbed to the surface of the modified electrode (Bard and Faulkner, 1980).

Association equilibrium constant and stoichiometric coefficients of the interaction of codeine and morphine with the dsDNA are described on the supplementary material file.

3.2.3. *Optimal reaction conditions*

As a second goal of this study, we investigated the possibility of employing the codeine and morphine–DNA interaction described above as an analytical tool for their determination using the DNA electrochemical biosensor. The experiments were conducted using the same reaction media employed in the previous sections, i.e., 0.10 mol L^{-1} phosphate with pH 7.0 stirred at 400 rpm. The codeine and its metabolite morphine were convectively accumulated on dsDNA/MWCNTs–PDDA/PGE surface due to their interaction with the dsDNA.

Fig. 4A shows the effect of accumulation time of codeine and morphine on their electrochemical signals which are characterized by a clear increase in the voltammetric signal up to 5 min. Consequently, this time (300 s) was selected as the accumulation time of codeine and morphine for further studies. A second parameter evaluated was the effect of the dsDNA concentration used to generate the modified electrode. Fig. 4B shows the effect of the dsDNA concentration for the both analytes. For morphine, an increase in the electrochemical response was observed between 0.6 and 0.8 mg mL^{-1} of the dsDNA concentration. Moreover, for codeine, an increase in the electrochemical response was observed between the dsDNA concentrations of 0.6 and 1.2 mg mL^{-1}. For comparative purposes, 1.0 mg mL^{-1} dsDNA concentration was selected for further experiments.

3.2.4. *Analytical performance*

The precision of the method was evaluated by repeating the experiments on the same days (within day reproducibility) with different standard solutions (freshly prepared at the same concentration). It was not possible to use the electrodes for more

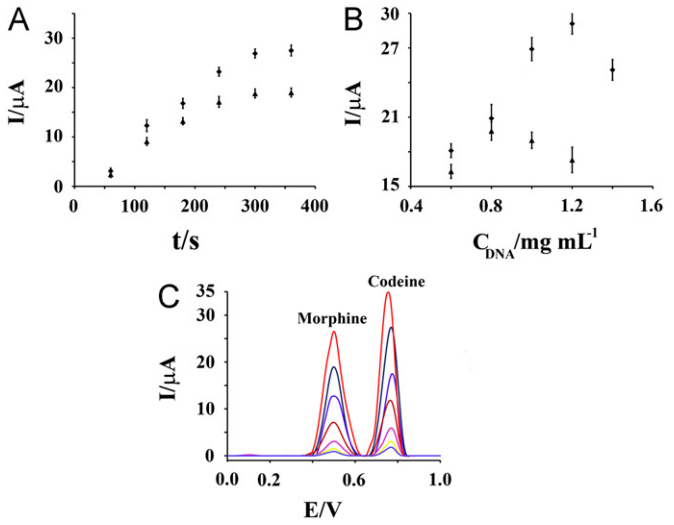

Fig. 4. (A) The effect of accumulation time on the electrochemical responses of codeine (♦) and morphine (▲) at dsDNA/MWCNTs–PDDA/PGE. (B) The effect of the dsDNA concentration on the electrochemical responses of codeine (♦) and morphine (▲) at dsDNA/MWCNTs–PDDA/PGE electrode, using 300 s as an accumulation time. (C) Differential pulse voltammograms for different concentrations of codeine and morphine at dsDNA/MWCNTs–PDDA/PGE after 300 s as an accumulation time. From up to down; 42.0, 30.0, 20.0, 10.0, 2.0, 0.8 and 0.05 μg mL^{-1} morphine and 40.0, 30.0, 20.0, 12.0, 4.0, 0.8 and 0.05 μg mL^{-1} codeine, respectively.

Table 1
Regression data of the calibration lines for determination of codeine and morphine.

	Codeine	Morphine
Linearity range (μg mL^{-1})	0.5–40	0.5–42
Number of point	11	11
Slope (μA mL μg^{-1})	0.7953	0.5937
Intercept (μA)	2.1899	1.098
Correlation coefficient	0.9977	0.9982
Detection limit (μg mL^{-1})	0.041	0.043
Limit of quantitation (μg mL^{-1})	0.137	0.143
RSD%	7.4	6.9

than one measurement and therefore, they had to be replaced each time. The reproducibility results (RSD%) ($n=5$) of the measuring current (after accumulation on dsDNA/MWCNTs–PDDA/PGE) were calculated as 7.4% and 6.9% for 1.0 mg mL^{-1} codeine and morphine, respectively. These results indicate that the whole protocol to determine codeine and morphine, using dsDNA/MWCNTs–PDDA/PGE, is reproducible.

The performance of the modified electrode was assessed by varying codeine and morphine concentrations. The peak current was increased linearly with codeine and morphine concentrations above the ranges of 0.05–40.0 μg mL^{-1} and 0.05–42.0 μg mL^{-1}, respectively (Fig. 4C and Fig S-3). Finally, detection limits of 0.041 and 0.043 μg mL^{-1} (calculated as three times of the standard deviation of the blank over sensitivity), and quantification limits of 0.137 and 0.143 μg mL^{-1} (calculated as ten times of the standard deviation of the blank over sensitivity) were obtained for codeine and morphine, respectively. The characteristics of the calibration plots are summarized in Table 1. These values of the detection limits and the linear dynamic ranges for codeine and morphine are comparable or even better than those obtained in several previous studies (Tables S-2 and S-3). This method, being fast, simple, sensitive, selective and cost effective, is used for recognition and evaluation of codeine and morphine.

Interference studies were carried out with several species prior to the application of the proposed methods for the assay of codeine and morphine in real samples. The potential interfering substances were chosen from the group of substances commonly found with codeine and morphine in biological fluids, pharmaceutical formulations and tablets. Tolerance limit defined as the maximum concentration of the potential interfering substance causes an error less than 3% for simultaneous determination of 1.0 μg mL^{-1} codeine and morphine. The results after the experiments revealed that 1000-fold of glucose, sucrose, lactose, fructose, citric acid, ascorbic acid, methanol, ethanol, Ca^{2+}, Mg^{2+}, SO_4^{2-}, Al^{3+}, NH_4^+, F^-, alanine, phenylalanine, methionine, glycine, glutamic acid, tryptophan, uric acid, aspirin, and thiourea and 600-fold of cysteine and cystine did not affect the selectivity. The obtained data showed that the proposed method is selective for simultaneous determination of codeine and morphine. The selectivity of the dsDNA-modified PGE to codeine and morphine is due to the insufficient interaction of other substances with the dsDNA in the selected conditions.

In order to investigate the accuracy of the method, urine, blood plasma, morphine sulfate injection solution, and expectorant cough syrup samples were analyzed to determine their codeine and/or morphine contents. In this regard, the samples were prepared as described in the sample preparation section. For morphine sulfate injection and expectorant cough syrup samples, the concentration of codeine and morphine in the samples was determined. Then, known amounts of both codeine and morphine were added to the samples and the analytes were measured again simultaneously using the biosensor by standard addition method. For blood plasma and urine samples, the concentration of codeine and morphine in the samples were determined. Then, known concentrations of codeine and morphine were added into the same samples volume before their being centrifuged. After samples preparation, the codeine and morphine in the spiked samples were measured simultaneously. Table 2 compares the results obtained from the proposed biosensor with those determined by standard GC–MS method (Dienes-Nagy et al. 1999). It should be noted that the results obtained using the proposed method are in good agreement with those obtained by the GC–MS method and with the data declared by the manufacturer. The statistical comparisons of the values obtained by these methods for the determination of codeine and morphine were made by student's *t*-test and the variance ratio of *F*-test. The experimental *t*-values (at $P=0.05$) for both techniques did not exceed the theoretical ones. These results indicate that the accuracy of the proposed methods is slightly better than that of the chromatographic method. In addition, the reference method is considerably more laborious (with respect to the preparation of a sample) than the developed methods. Therefore, the proposed methods could be successfully applied for codeine and morphine assay in syrup dosage form without any interference.

4. Conclusion

The data presented in this study led us to the conclusion that even low amounts of codeine and morphine in the dsDNA solution produce appreciable changes in conformation of the dsDNA, as indicated by DPV response. In solution, the drugs, codeine and morphine, can bind to the dsDNA with a binding constant of $4.40(\pm0.26)\times10^4$ for codeine and $4.60(\pm0.36)\times10^5$ for morphine, as determined in this work by voltammetric method. Interactions between codeine and morphine with surface-bonded DNA were further studied using the dsDNA/MWCNTs–PDDA/PGE. The results revealed that the principal interactions for codeine and morphine were electrostatic and intercalation modes, respectively. The prepared DNA/PGE was successfully applied in the estimation of codeine and morphine content in commercial dosage forms and human body fluids. Short pre-concentration time permits convenient measurements of low concentrations of these drugs. The proposed electrochemical stripping method, using dsDNA modified PGE couple, has

Table 2
Analytical results of spiked syrup, injection drug, urine and plasma.

Sample	Added ($\mu g\ mL^{-1}$)		Found[a] ($\mu g\ mL^{-1}$)		GC–MS[a] ($\mu g\ mL^{-1}$)		F_{ex}		F_{tab}	t_{ex}		t_{tab}
	Codeine	Morphine	Codeine	Morphine	Codeine	Morphine	Codeine	Morphine		Codeine	Morphine	
Expectorant codeine syrup[b]	–	5.00	4.68 ± 0.42	4.75 ± 0.38	4.75 ± 0.51	4.70 ± 0.43	1.5	1.3	6.8	1.1	0.4	2.3
	5.00	3.00	9.80 ± 0.90	2.84 ± 0.28	9.81 ± 0.83	2.80 ± 0.30	1.2	1.1	6.8	0.4	0.6	2.3
Morphine injection solution[c]	2.00	–	2.11 ± 0.30	5.30 ± 0.67	2.08 ± 0.25	5.25 ± 0.51	1.4	1.7	6.8	1.0	0.9	2.3
	4.00	5.00	5.32 ± 0.55	9.50 ± 0.87	5.41 ± 0.65	9.78 ± 0.70	1.4	1.5	6.8	1.2	1.9	2.3
Urine	–	–	Less than detection limit	Less than detection limit	–	–						
	1.00	1.00	0.96 ± 0.09	1.06 ± 0.11	0.96 ± 0.10	1.03 ± 0.09	1.2	1.5	6.8	0.2	0.6	2.3
	5.00	8.00	5.21 ± 0.53	7.79 ± 0.64	5.32 ± 0.68	7.50 ± 0.56	1.6	1.3	6.8	1.4	0.9	2.3
Plasma	–	–	Less than detection limit	Less than detection limit	–	–						
	1.00	1.00	1.08 ± 0.08	1.06 ± 0.09	1.04 ± 0.10	1.04 ± 0.11	1.6	1.5	6.8	0.4	0.4	2.3
	5.00	8.00	5.11 ± 0.50	7.89 ± 0.55	5.24 ± 0.86	7.76 ± 0.49	2.9	1.2	6.8	1.7	1.0	2.3

[a] Each value is the mean of five replicate experiments.
[b] Each 5.0 mL contains: Codeine phosphate 10 mg, Guaifenesine 100 mg, Phenylpropanolamine.HCl 12.5 mg, Sodium saccharine 7.5 mg, Ethanol 150 mg; (Darou Pakhsh Co., Iran).
[c] Each milliliter contains: Morphine sulfate 0.5 mg, and sodium chloride 9.0 mg in water.

the advantages of extremely low detection limits and suitability for online and in situ measurements. The proposed technique and its applications represent a good alternative for the detection and determination of codeine and morphine in pharmaceutical dosage forms and human body fluides. This electroanalytical method represents a good alternative for quality control since the preparation of the sample is easy and the excipients do not interfere with the determination. Consequently, separation, filtration, extraction or evaporation procedures are not needed. Further developments in the electrochemical DNA biosensor techniques will enable researchers to screen a large number of new drug compounds for their antiviral activity, which may ultimately lead to the design of efficient and effective drugs with fewer side effects. These results are expected to offer new insights into the study of the interactions of drugs with DNA.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2012.09.039.

References

Ahsanul, H., Stewart, J.T., 1999. Journal of Liquid Chromatography and Related Technologies 22, 1193–1204.
Bard, A.J., Faulkner, L.R., 1980. Electrochemical Methods: Fundamentals and Applications. John Wiley & Sons, New York.
Berga, T., Lundanes, E., Christophersen, A.S., Strand, D.H., 2009. Journal of Chromatography B 877, 421–432.
Bogusz, M.J., Maier, R.D., Erkens, M., Driessen, S., 1997. Journal of Chromatography B 703, 115–127.
Brewer, W.E., Galipo, R.C., Sellers, K.W., Morgan, S.L., 2001. Analytical Chemistry 73, 2371–2376.
Carter, M.T., Rodriguez, M., Bard, A.J., 1980. Journal of the American Chemical Society 111, 8901–8911.
Coles, R., Kushnir, M.M., Nelson, G.J., McMillin, G.A., Urry, F.M., 2007. Journal of Analytical Toxicology 31, 1–14.
Dienes-Nagy, A., Rivier, L., Giroud, C., Augsburger, M., Mangin, P., 1999. Journal of Chromatography A 854, 109–118.
Elsayed, M.A.H., Belal, S.F., Elwalily, A.F.M., Abdine, H., 1979. Analyst 104, 620–625.
Ensafi, A.A., Heydari-Bafrooei, E., Amini, M., 2012a. Biosensors and Bioelectronics 31, 376–381.
Ensafi, A.A., Rezaei, B., Amini, M., Heydari-Bafrooei, E., 2012b. Talanta 88, 244–251.
Erdem, A., Kara, P., Kerman, K., Ozkan, D., Ozsoz, M., 2003. Electroanalysis 15, 613–619.
Fojta, M., Jelen, F., Havran, L., Palecek, E., 2008. Current Analytical Chemistry 4, 250–262.
Garrido, J.M.P.J., Delerue-Matos, C., Borges, F., Macedo, T.R.A., Oliveira-Brett, A.M., 2004a. Electroanalysis 16, 1427–1433.
Garrido, J.M.P.J., Delerue-Matos, C., Borges, F., Macedo, T.R.A., Oliveira-Brett, A.M., 2004b. Electroanalysis 16, 1419–1426.
Ghica, M.E., Pauliukaite, R., Fatibello-Filho, O., Brett, C.M.A., 2009. Sensors and Actuators B 142, 308–315.
Greenway, G.M., Nelstrop, L.J., Port, S.N., 2000. Analytica Chimica Acta 405, 43–50.
He, H., Shay, S.D., Caraco, Y., Wood, M., Wood, A.J.J., 1998. Journal of Chromatography B 708, 185–193.
Hofmann, U., Seefried, S., Schweizer, E., Ebner, T., Mikus, G., Eichelbaum, M., 1999. Journal of Chromatography B 727, 81–88.
Ibrahim, M.S., 2001. Analytica Chimica Acta 443, 63–72.
Idris, A.M., Alnajjar, A.O., 2008. Talanta 77, 522–526.
Meatherall, R., 1999. Journal of Analytical Toxicology 23, 177–186.
Pournaghi-Azar, M.H., Saadatirad, A., 2008. Journal of Electroanalytical Chemistry 624, 293–298.
Pournaghi-Azar, M.H., Saadatirad, A., 2010. Electroanalysis 22, 1592–1598.
Raffa, R.B., 1996. The American Journal of Medicine 101, 40S–46S.
Ramos Martos, N., Molina Díaz, A., NavalÓn, A., Capitán-Vallvey, L.F., 2001. Analytical Letters 34, 579–595.
Salimi, A., Hallaj, R., Khayatian, G., 2005. Electroanalysis 17, 873–879.
Shih, Y., Zen, J.M., Yang, H.H., 2002. Journal of Pharmaceutical and Biomedical analysis 29, 827–833.
Watson, D.G., Su, Q., Midgley, J.M., Doyle, E., Morton, N.S., 1995. Journal of Pharmaceutical and Biomedical analysis 13, 27–32.
Xu, F., Gao, M.N., Wang, L., Zhou, T.S., Jin, L.T., Jin, J.Y., 2002. Talanta 58, 427–432.
Zen, J.M., Chang, M.R., Chung, H.H., Shih, Y., 1998. Electroanalysis 10, 536–540.
Zhang, X.X., Li, J., Gao, J., Sun, L., Chang, W.B., 2000. Journal of Chromatography A 895, 1–7.
Zhou, T., Yu, H., Hu, Q., Fang, Y., 2002. Journal of Pharmaceutical and Biomedical Analysis 30, 13–19.