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Simultaneous DPV determination of morphine and codeine using dsDNA modified screen printed electrode strips coupled with electromembrane extraction

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ABSTRACT

In this work a sensitive electrochemical sensor for simultaneous determination of morphine and codeine constructed by application of disposable screen printed carbon electrode strips (SPCE) modified by double strand (ds) calf thymus DNA. According to the results of the modified SPCE strips and experimented parameters, we observed a considerable shift between potentials of morphine and codeine current peaks. Related to these observed shifts, we studied on the effect of the concentration of modifier and pH value on the anodic oxidation pattern of morphine and codeine in the case of optimize the method to get better signals with maximum potential distance. Also to boosting the LODs of this electroanalytical method coupled with an electro-membrane preconcentration (EME) step. The calibration curve which was plotted by the variation of differential pulse voltammetry (DPV) currents as a function of different morphine and codeine concentration were linear within the range of 0.7– 40 µM and 2.3- 40 µM for morphine and codeine respectively. Also the limits of detection were 0.07 µM and 0.23 µM, respectively. Finally, the proposed method was able to determine morphine and codeine simultaneously and effectively in urinary real samples.

Keywords: Differential Pulse Voltammetry (DPV), Codeine, morphine, DNA

INTRODUCTION

Morphine and Codeine (Fig. 1) are pain medications of the opiate type. These chemical compounds are main alkaloids in poppy seeds, and have pharmacological and toxicological activity [1]. In addition to their pharmacological applications, morphine and codeine abuse for illegal is one of the most serious forensic problems of recent two decades. In this context, drug analysis and determination plays an important roles in drug quality control, pharmaceutical researches and forensic science and therefore, efforts in the case of developing simple, sensitive, and accurate methods for the determination of this chemical compounds in real sample is very important and critical. 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[2].

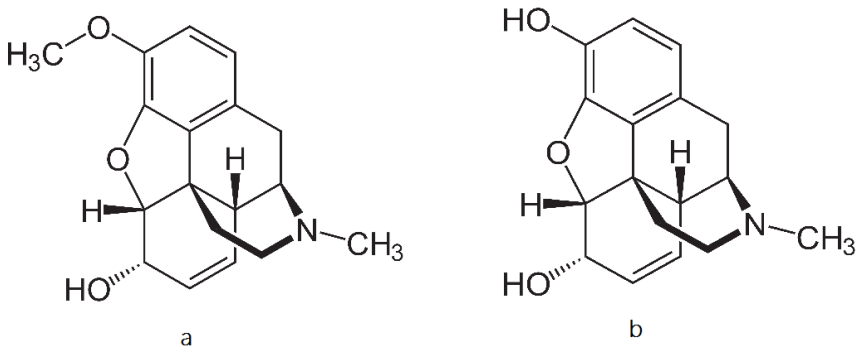


Fig. 1: Chemical structure of (a) codeine and (b) morphine

By today, several reports have been reported about application of analytical techniques and methods for detection and determination of these compounds such as high performance liquid chromatography [3-5], gas chromatography [6, 7], thin-layer chromatography [8], capillary electrophoresis [9, 10] and spectroscopy [11]. These methods are very powerful and accurate, and even some of them are the standard techniques for determination procedure of these types of drugs, but in some cases they are also very expensive, time consuming and requires a very complicated pretreatment steps.

However according to the ongoing needs to development of new, simple, fast and reasonably price analytical methods for determination illegal or legal drugs in pharmaceutical industries, forensic science and scientific researches, electroanalytical methods have attracted more attention. In particular in this area, the uses of modified electrodes for the sake of increasing the sensitivity and selectivity have been recently recommended. In this subject, use of electroactive compounds with electrostatic binding ability to the analytes could be effective on the electrochemical behavior of the analytes and might let us to achieve more sensitive and specific analytical methods. According to the Ensafi et al studies [2] the electroactivity of nucleic acids has allowed the development of more sensitive and rapid electrochemical techniques.

Using a full range of physical and biochemical methods, studies have now established double helical DNA as a medium for the efficient transport of electrons and electron holes. [12, 13]. Electron conductivity was clearly demonstrated in recent experiments on aligned DNA films, and this conductivity was found only along the direction parallel to the helical axis [12]. The charge transfer phenomenon in the DNA strain could be explained by Tunneling theory. Based on this theory charges are hopping between discrete base orbitals (Fig. 2) [13].

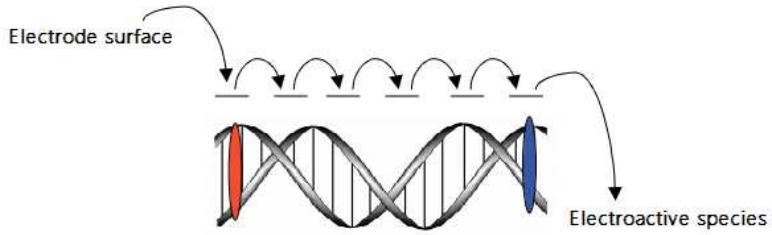


Fig. 2: Charge traveling from electrode to electroactive compound by hopping between discrete molecular orbitals on the DNA skeleton

In the case of DNA electrochemical biosensors The DNA-trapped compounds can either be detected directly if they are electroactive molecules or via changes in electrochemical DNA signal [13-17]. DNA association interactions are of interest for chemistry, molecular biology, and medicine, particularly for drug discovery and environmental/medical processes [18, 19]. They concern association with both inorganic and organic compounds as well as various types of assisted interactions such as metal and metal complex–DNA chemistry. These interaction which results in detection/preconcentration of analytes on the DNA biosensors surfaces are mainly noncovalent host-gust interaction and are represents generally by: (a) Intercalation between the stacked base pairs of dsDNA, (b) binding at major or minor grooves of the DNA double helix (electrostatic interactions) [20].

In the past decade, the study of DNA-mediated charge transport has been of great interest. While the finer kinetic and mechanistic issues surrounding DNA-mediated charge transport chemistry are still being debated, it has become apparent that these reactions are extremely sensitive to DNA π-stacking [21].

Intercalation and groove binding are the two most common modes by which small molecules bind directly and selectively to DNA. Intercalation, which is an enthalpically driven process, results from the insertion of a planar aromatic ring system between DNA base pairs with concomitant unwinding and lengthening of the DNA helix. In contrast, groove binding, which is predominantly entropically driven, involves covalent or non-covalent (electrostatic) interactions that do not perturb the duplex structure to any great extent [22].

In this study we report a DNA modified screen printed electrode (SPE) biosensor based on differential pulse voltammetry (DPV) technique (Fig. 3) coupled with electro membrane microextraction (EME) technique for simultaneously determination of morphine and codeine in urine samples.

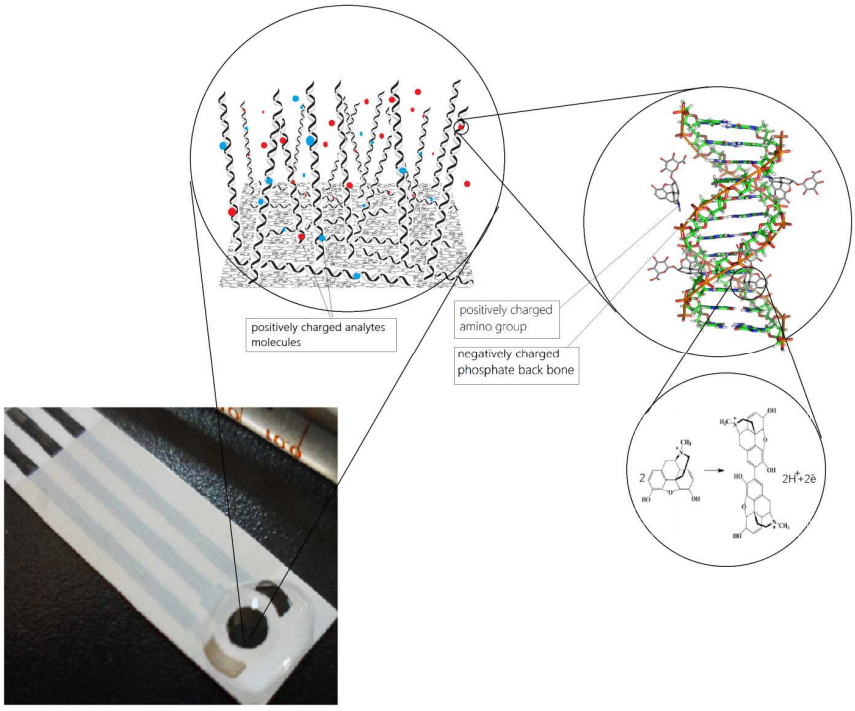


Fig. 3: A schematic diagram for potential sensing mechanism of proposed dsDNA biosensors

In the case of DNA biosensor we apply an optimization strategy for DPV simultaneous determination of morphine and codeine based on different electrochemical behaviors of these compounds in the existence of immobilized dsDNA on the surface of electrodes and various pH values.

EME technique is a selective extraction method which is perform by applying an electrical voltage across the supported liquid membrane (SLM) [23]. In EME, the ionized target compound(s) migrate from an aqueous sample, donor phase, through an organic solvent located in the pores of a porous hollow fiber into an aqueous acceptor phase inside the lumen of hollow fiber [24]. The chemical nature of the SLM is highly critical for a successful and selective electrokinetic crossmembrane extraction. Basic analytes are successfully extracted with nitro aromatic solvents like 2-nitrophenyl octyl ether (NPOE) [23],while, acidic compounds are extracted by aliphatic alcohols like 1-octanol [25]. It has also been demonstrated that the selective extraction of polar drugs in the presence of non-polar ones can be improved by means of applying carriers in the composition of SLM [26]. Thus, selective extraction can be obtained if an appropriate composition of SLM is chosen and regarding to our analytes we adopted an optimized composition of di-(2-ethylhexyl) phosphate as the anionic carrier and 2-nitrophenyl octyl ether as the organic solvent from similar reported study [27].

MATERIALS AND METHODS

Experimental

Reagents and materials: All reagents used were of analytical grade. Potassium monohydrogen phosphate, potassium dihydrogen phosphate, perchloric acid, Acetic acid, Sodium acetate, $K_3Fe(CN)_6$, $K_4Fe(CN)_6$, NaOH and HCl were obtained from the Merck Company (Darmstadt, Germany). 1-octanol and di-(2-ethylhexyl) phosphate as the supported liquid membrane, purchased from Fluka (Buchs, Switzerland). dsDNA salt was purchased from Sigma (St. Louis, USA) and was dissolved in 0.1 mol L^{-1} phosphate buffer solution (prepared using KH_2PO_4 and K_2HPO_4, pH=5.0) and was kept in refrigerator at 4°C. Morphine and Codeine phosphate were kindly donated by applied research center of anti narcotic police of Iran. All other chemicals were of analytical reagent grade and double distilled water (DDW) was used throughout.

Apparatus and instrumentations: CVs and DPVs were performed with a Palm Sens (Palm Instruments BV, Netherland). Three-electrode screen-printed electrochemical strips were provided by DropSens (Oviedo, Spain). The extractions were done by using a HY3005F D.C power supply (Hua Yi Electronic, China) with programmable voltage within the range of 0–400 V and current range of 0–5 A. The electrodes used in EME procedure were platinum wires with diameters of 0.2 mm and 0.5 mm for cathode and anode, respectively, and were obtained from Pars Pelatine (Tehran, Iran). The porous hollow fiber used for immobilizing the SLM and housing the AP was a PP Q3/2 polypropylene (Membrana, Wuppertal, Germany) with an internal diameter of 0.60 mm, 200 mm wall thickness and 0.2 mm pores.

Preparation of the biosensor (DNA/SPCPE): The SPCE surface was pretreated by applying a potential of +1600 mV for 120 s and +1800 mV for 60 s in 10 mL freshly prepared acetate buffer, under stirred conditions; this procedure was necessary to oxidize all contaminants present on the electrodes surface and also to activate the carbon ink surface and make it more hydrophilic and susceptible to the dsDNA immobilization[28]. In the proposed condition DNA adsorption was performed by electrostatic adsorption mainly from one end of helical strand [29, 30]. The electrochemical strip was then immersed in the DNA solution for immobilization onto the screen-printed electrode surface at fixed potential of +0.5 V vs Ag screen-printed pseudo-reference electrode for 120 s under stirring. After that a cleaning step was performed by immersion of the strip in a clean acetate buffer solution, at open-circuit for 30 s.

Preparation of real samples: A total volume of 50 ml urine samples were collected from 3 young persons who were not consumed codeine or morphine at all in recent 10 days. Urine samples were stored in refrigerator immediately after collection.

Electrochemical measurements: In all electrochemical measurements the SPCE strips were first activated in the blank solution (NaOH 0.1 M (20 ml),HCl 0.1 M (20 ml)) by cyclic voltammetric sweeps between 0 mV and 600 mV. The intended solvent with specific amount of analytes and pH were introduced to the working electrode as a droplet (60 uL). Unless stated, otherwise scan rate 50 mV s^{-1} and potential step (E_{step}) 5 mV were used in CV measurments. The pulse amplitude (E_{amp}) +50 mV (anodic scan) or −50 mV (cathodic scan), pulse width 100 ms, scan rate 20 mV s^{-1} , and E_{step} 5 mV were used in DPV.

Procedure for EME: Three milliliters of sample solution containing the analyte with a determined pH was transferred into the sample vial. To saturate the pores of hollow fiber wall by the organic solution, 7 cm piece of hollow fiber was cut out and dipped in the 1-octanol solution containing 0.1 V/V di-(2-ethylhexyl) phosphate for 5 s and then the excess of organic solution was gently wiped away by blowing air with a medical syringe. The upper end of hollow fiber was connected to a medical needle tip as a guiding tube which was inserted through the rubber cap of the vial. 15 µl of 100 mM HCl (acceptor solution) was introduced into the lumen of the hollow fiber by a microsyringe and the lower end of hollow fiber was sealed with a small piece of aluminum foil. One of the electrodes, the cathode, was introduced into the lumen of the fiber. The fiber containing the cathode, SLM and the acceptor solution was afterward directed into the sample solution. The other electrode, the anode, was led directly into the sample solution. The electrodes were subsequently coupled to the power supply and the extraction unit was placed on a stirrer with stirring speed of 1000 rpm. The power supply was turned on and extraction was performed for 15 min. After the extraction was completed, the acceptor solution was collected by a microsyringe and after pH adjustments, transferred to the SPCE surface for further electromeasurments.

RESULTS AND DISCUSSION

Characteristics of modified SPCE with dsDNA by Cyclic-voltammetry of $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$: The modification step could be investigated based on study of electron transfer of ferricyanide through the modified electrodes. As it represented in Fig. 4, cyclic voltammograms obtained for modified and unmodified SPCEs in 1 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M PBS solution (pH 5.0, 0.1 M KNO_3) at the scan rate of 50 mV/S [31]. A pair redox peaks can be seen at the bare SPCE (fig 4-a). These peaks could be definitely assigned to the redox behaviors of $[Fe(CN)_6]^{3-/4-}$. In the dsDNA modified SPEs there are significant decreases of the peak height and increases of the peak to peak potential separation. This induced changes are significantly related to the immobilized amount of DNA on the surface of working electrode and could indicated that the dsDNA has been successfully immobilized on the working electrode surface. The peak current decrease could be well assigned to the repellence of $[Fe(CN)_6]^{3-/4-}$ by the negatively charged phosphate backbone of DNA [22].

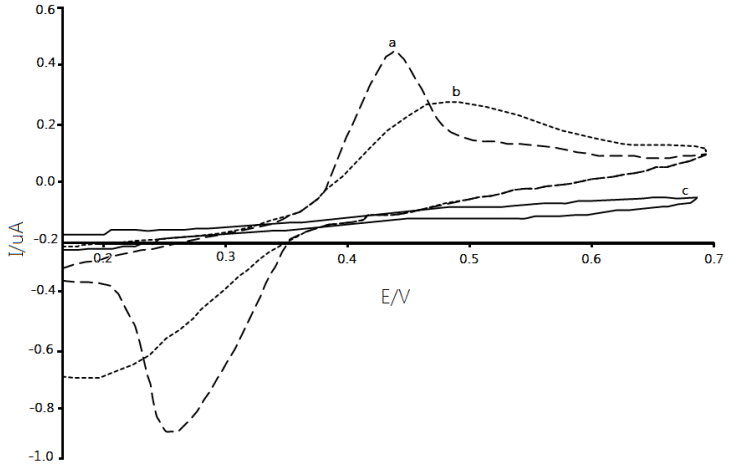


Fig. 4: Cyclic voltammograms of the bare SPCE (a) modified SPE with immobilized dsDNA (0.4 mg ml^{-1}) (b) and (1 mg ml^{-1}) (c) in 1M $[Fe(CN)_6]^{3-/4-}$ and 0.1M KNO_3 at the scan rate of 50 mV/S

Electrochemical behavior of codeine and morphine investigation with dsDNA modified SPCE strips: To study the electrochemical reaction of morphine and codeine on the surface of modified and unmodified SPCE we applied CV measurements. The cyclic voltammograms of SPCE in the absence and presence of 0.85 µM morphine and 0.90 µM codeine are shown in Fig.6. These CV's were recorded at pH of 5. As it is represented in figure 5, a modified SPE with pure dsDNA displays no electrochemical signals under the experimental conditions and applied potential range. This is mainly due to the merging of the anodic peak at low amount of immobilized dsDNA and the background discharge [32] (Fig. 6-a). In the other hand, three oxidation peaks at 0.39 V (O_I), 0.68 V (O_{II}) and 0.82 V (O_{III}) were obtained for the SPCE after the addition of morphine and codeine. There is no significant cathodic peak observed.

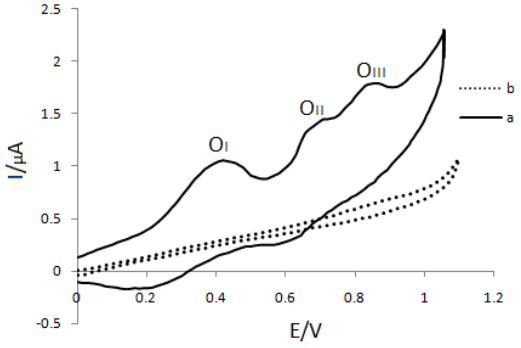


Fig. 5: The cyclic voltammograms of SPCE in the absence and presence of 0.85 µM morphine and 0.90 µM codeine in pH 5

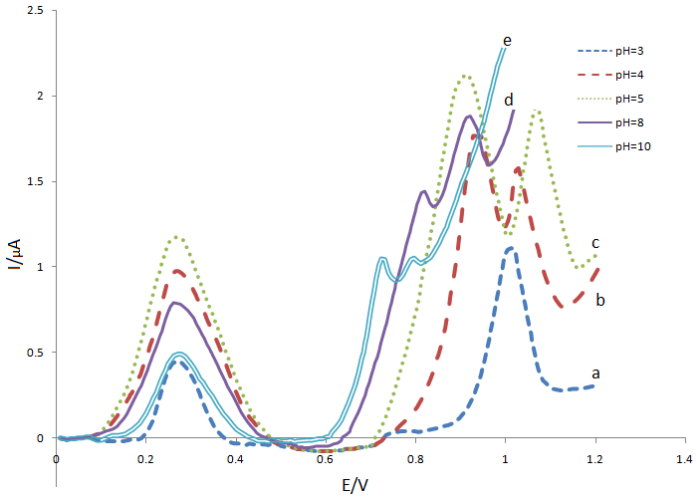


Fig 6: Differential pulse voltammograms of 0.9 µM codeine and 0.85 µM morphine in different buffer solution with pH intervals from 3 to 10

Codeine is a complex molecule and fairly stable in the solid state, but in aqueous solutions degrades relatively rapidly in the presence of strong acids and bases. In this degradation process, isomerization reactions play an important role and it is closely depended to the pH [33]. Anodic oxidation of codeine solution follows a complex mechanism according to its degradation procedure. In the case of investigation of pH effect on codeines oxidative pathways a detailed differential pulse voltammetric measurement was carried out. As shown in Fig. 6, by changing the pH from 3 to 10 the anodic oxidation pathway of codeine changes dramatically. At pH 4 there are two well-defined anodic peaks for codeine, E_p=0.99 V and E_p=+1.05 V (Fig. 6-a); these waves should result from oxidation of the tertiary amine and 6-hydroxy groups [34] . In fact, the decrease of peak potential with increasing pH observed for the oxidation of the tertiary amine group together with the pH independent potential corresponding to oxidation of the hydroxy group until pH 5 means there is a separation between the two peaks[34]. Based on these explanations, we can say that the anodic wave observed at pH 2, E_p=1.09 is might be a result of the sum of two close peaks related to oxidation of the hydroxy and tertiary amine functional groups. The identification of these two anodic waves, involving the tertiary amine and 6-hydroxy group, was confirmed by Brett et al [34]. At pH 8.0, three anodic peaks for codeine could be observed (Fig. 6-d). The appearance of a new wave can be attributed to the oxidation of the 3-methoxy group [2]. Finally at the pH 10 there are four anodic waves are observed (Fig. 6-e). A small shoulder is seen near the second peak, which means that probably the second peak is, for lower pH, also the result of the sum of two anodic waves. This new peak, is probably related with a subsequent oxidative process involving the secondary amine group from the oxidation of the tertiary amine group present in codeine [34].
In the other hand, morphine was electrochemically oxidized with one anodic peak in the entire applied pH interval at E_p=+0.25 V. This peak might be related to the oxidation of the phenolic group [34]. As it obvious in Fig. 5, the highest DPV peak for morphine in the pH interval is belong to pH 5.

Proton is always involved in the electrochemical reaction of organic compounds and exerts significant impact on the reaction speed. Decrease in Morphine oxidation signal at lower and higher pHs can be related to the devaluation of electrostatic interaction between dsDNA and morphine, because protonation of phosphate groups of DNA in lower pH or diminish the positive charge of morphine at higher pH [22].

In the case of study the effect of the immobilized amount of dsDNA on DPV pathway of morphine and codeine some SPCE strips were prepared by different concentration of dsDNA. As it displayed in the Fig. 7, the maximum currents of the codeine anodic oxidation are shifted to positive potentials. Also the maximum current of DPVs is changed with the dsDNA concentration; the best current is belonging to the SPCE strip modified with 5mg ml^{-1}DNA solution. 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 [2]. The shifting to positive potentials might be the result

of merging two anodic peaks of codeine 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 [35].

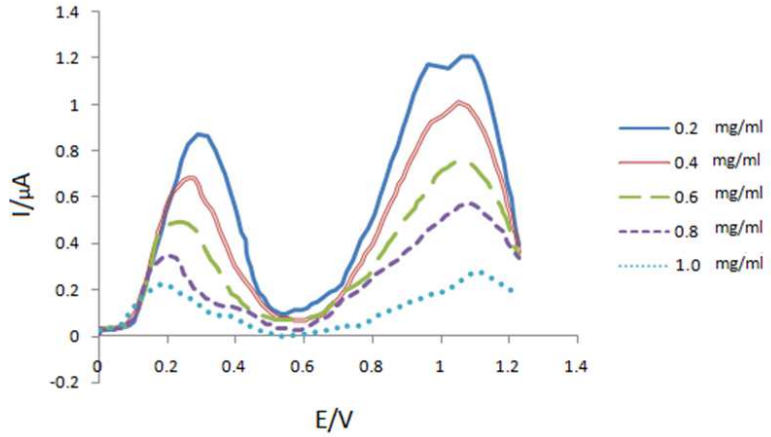


Fig. 7: Differential pulse voltammograms of 0.9 µM codeine and 0.85 µM morphine (pH= 5) for different SPCE strips modified by 0.2, 0.5, 0.8, 1.0 and 1.2 mg ml^{-1} dsDNA solution

As shown in Fig. 7, the oxidation signal of morphine is decreased with increasing the dsDNA concentration. Also there are obvious negative changes in the peak potentials by increasing the DNA concentration. It seems that the decrease of the oxidation signals is caused by decrease of the diffusion coefficient. Additionally, in the studied pH condition both morphine and codeine according to their pK_as (8.2 and 8.15 respectively) are charged. Based on Fig. 7, by increasing the immobilized dsDNA amount on the surface of the SPCE, the codeine peaks are shifting negatively. Also the behavior of the morphine peaks is completely different and they are shifting positively. According to the literatures, the negatively shifted peak potential is characteristic of an electrostatic mode of interaction and positively shifting is a result of a intercalative interaction behavior [35]. It may be claimed that codeine is bound to DNA mainly through an electrostatic mode and morphine interaction with DNA is based on intercalative interaction mode.

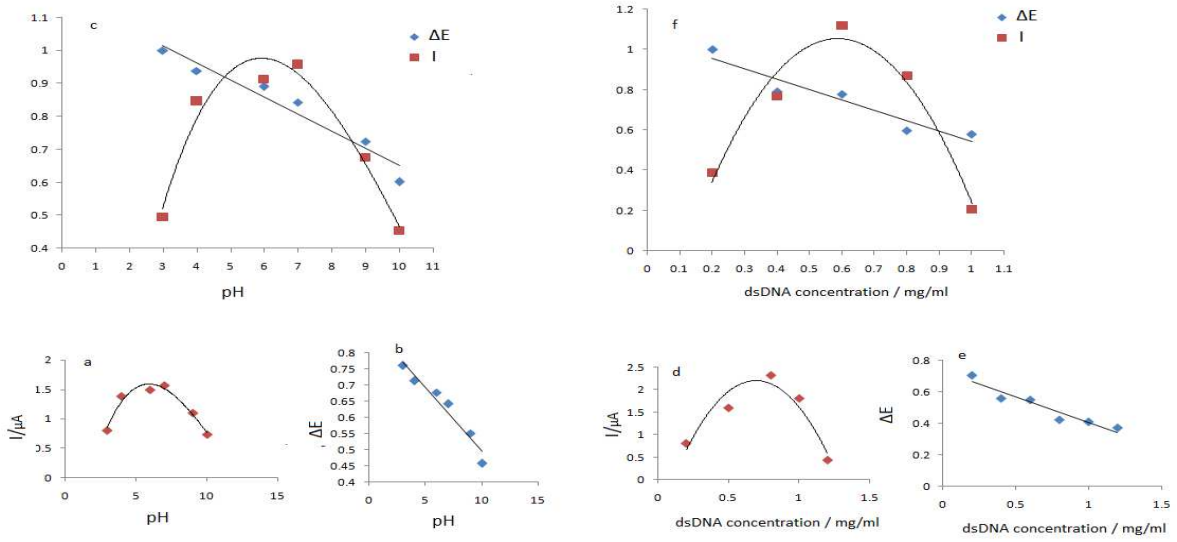


Fig. 8: a) plot of I vs. pH, b) plot of dE vs. pH, c) a and b in percentage scale for optimizing pH, d)plot of I vs. dsDNA concentration, e) plot of dE vs. dsDNA concentration and f) d and e in percentage scale for optimizing the dsDNA concentration optimizing

Optimization strategy: To achieve the best condition for simultaneous determination of morphine and codeine we considered two important parameters, the maximum potential space between morphine and codeine DPV peaks to reduce the potential of interference in higher concentrations, and the best current signals, in the case of improving the LODs. According to the previous section to get the best situation we controlled these parameters by applying pH adjustment and different immobilized dsDNA amounts. As it represented in the Fig. 8 the best optimized state for pH and dsDNA amount is 5 and 0.4 mg ml^{-1} respectively.

EME optimization: In order to optimize the EME condition we investigated four effective parameters on the extraction procedure including donor phase pH, applied potential, extraction time and hollow fiber length. In this case we got the sum of peak heights as the numerical response. As it represented in figure 7, the pH of the donor phase was investigated between 2.5 and 7.0 to determine the optimum pH condition for simultaneous extraction of morphine and codeine. The results are summarised in Fig. 8. The variations of total current by pH changes had a maximum point in pH=5. In this pH the positively charged form of both analytes are predominate and the majority of the analytes molecules could transfer from donor to phase SLM and subsequently transfer to the acceptor phase through the electrical field. In the lower pH amounts the higher concentration of proton can interfere in the oxidation mechanism of analytes and hinder the signals height.

In EME, the electrokinetic migration of the analytes across the SLM into the acceptor solution is greatly dependent upon the applied voltage. In a recent paper, Kjelsen et al. reported that the flux of analytes is affected by the magnitude of the applied voltage [36]. Increases in voltage causes an increases in the number of ions crossing through the membrane, as it shown in Fig. 9 the responses were increased until the voltage of 40 v. The unpleasant decrease in the total current after this point could be caused by creation of hydrogen gas bubbles inside the hollow fiber lumen as products of uncontrolled electrolysis of water. Also, the generated heats due to the increased number of crossing molecules through the membrane in higher voltages leads to loss of membrane organic layer as a function of time and subsequently decrease the extraction yields.

Extraction time is a very critical parameter in the presented extraction procedure. By increasing the extraction time the transferred molecules population of the target analytes from the sample solution to the SLM (in the pores of the fiber) and, subsequently, from the organic solvent to acceptor phase increases. On the other hand, if the extraction time is long, solvent losses and formation of air bubbles may occur, which would compromise the extraction. As it presented in Fig. 9 the responses were increased by increasing the extraction time till 15 minutes. This point is the equilibrium condition of the mass transfer rate between SLM, donor phase and acceptor phase.

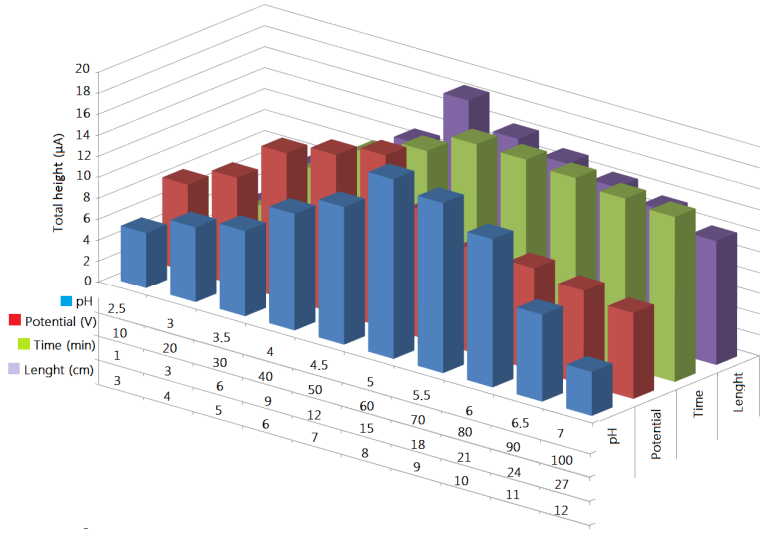


Fig. 9: Simultaneous investigation of pH, applied potential, Time and hollow fiber length effect on the responses

According to the constant diameter of lumen of the hollow fiber the length of HF is an indicator of the acceptor solvents volume. Generally in the hollow fiber based microextraction methods, a smaller volume of acceptor phase

causes a higher analyte concentration in that phase [37]. However, the acceptor phase volume should be large enough to promote analyte transport to the acceptor phase [38]. As seems in Fig. 8 the response amounts increases with HF length in the range of 3–7 cm, whereas further increase of HF length results in decrease of total current due to dilution effect. Based on results the optimum points for all four investigated parameters are represented in table 1.

Table: 1. The optimum condition for EME procedure

Parameter	pH	Applied potential (V)	Time (min)	Hollow fiber length (cm)
Optimized level	5	50	15	7

Simultaneous Differential Pulse Voltammetry determination of morphine and codeine: Under optimized conditions, simultaneous deferential pulse voltammetric determination of morphine and codeine has been carried out on the SPCE for mixture of extracted morphine and codeine with same concentration from blank urine matrix by means of EME method. The results show that the peak potential separation of 0.75 V and the peak height of morphine or codeine in the presence of different concentration of each other is linearly dependant only on their concentrations (Fig. 10).

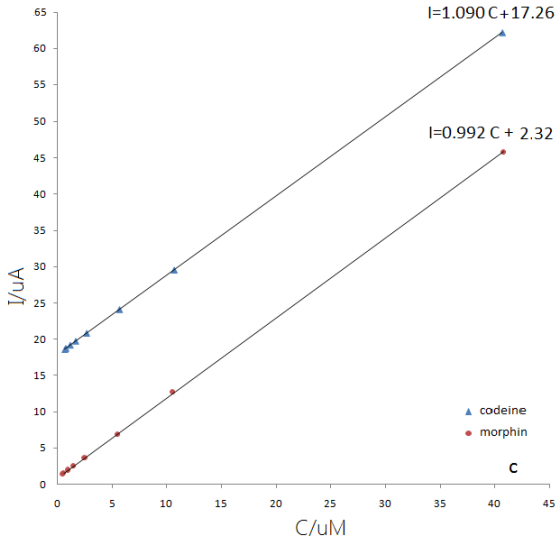


Fig. 10: Plots of I_p vs. morphine and codeine concentrations in the simultaneous determination after EME procedure (under optimized pH and modifier concentration)

Table: 2. Comparisons of various electroanalytical methods proposed for detection of morphine and codeine

Working electrod	Method	Linear range for morphine (µM)	LOD for morphine (µM)	LOD for codeine (µM)	Linear range for codeine (µM)	Reference
Glassy Carbon Electrode/ MWCNTs[a]	SWVb	0.5–150	0.2	-	-	[39]
PB/Pd-Al[c]	DPV	10-50	3	-	-	[40]
PSi/Pd–NS/CNTPE[d]	DPV	-	-	1–700.0	0.3	[41]
Boron doped diamond	DPV	-	-	0.01 – 0.1	0.8	[42]
PB/Pd-Al	hydrodynamic amperometry	2–30	0.8	2–30	0.8	[43]
modified-palladized aluminum electrode	CV-amperometric	-	-	2–50	0.8	[44]
dsDNA/SPCE	EME-DPV	0.25-40	0.07	0.9-40	0.26	This work

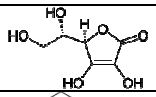
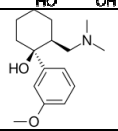
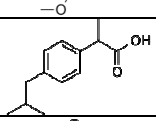
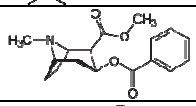
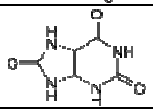
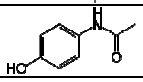
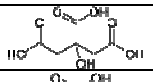
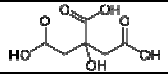
[a]Multiwalled carbon nanotube ; [b]Square wave voltammetry; [c]Prussian blue film modified-palladized aluminum electrode
[d]pencil graphite electrode modified by double strand DNA, Poly(diallyl dimethyl ammonium chloride) and multiwall carbon nanotubes

Figure of merit and analytical performance: The optimized EME configuration which was adopted in this work performed a very well extraction for only the positively charged compounds with logarithmic molecular lipophilicity (log p) values lower than 1.4 (the log p of morphine and codeine are 0.89 and 1.19 respectively). It's mean that the neutral and negative compounds remain in the sample matrix and cannot be transferred into the acceptor phase. The detection limit of the method (LOD) for morphine and codeine, from the first range of the calibration curve, was calculated to be 0.07 µM and 0.23 µM respectively. In the case of comparison the proposed method with previously similar works showed that the EME–dsDNA/SPCE-DPV method has a reasonable lower detection limit, an acceptable linear dynamic range, better selectivity, more effective cleanup, lower cost and easier operation of the detection system (table 2).

Selectivity of the method: In the case of removing the potential electroactive interference compounds in the real samples we applied a clean-up procedure coupled with the DPV technique. The main parameters affecting the EME selectivity are the SLM composition, log p of the analyte, applied extraction voltage and extraction time. Optimal conditions can be used in order to increase the selectivity of extraction [24]. According to the optimum condition of EME procedure which were applied in this work, the extraction step lead to specific extraction of the positively charged compounds with log p values lower than 1.5. Hence, the negative and neutral drugs remain in the sample solution and cannot be transferred into the acceptor phase.

In order to investigate the selectivity of the method some common commercial drugs and natural urinary compounds (Table 3) which are electroactive compounds and has great potential to be a major interference in the DPV measurements had been selected and investigated.

Table: 3. List of investigated electroactive potential interferes

Name of drug	Chemical structure	Log p	pKa
Vitamine C		-1.85	4.7
Tramadole		2.4	9.41
Ibuprofen		3.97	4.91
Cocaine		2.30	8.61
Uric acid		-2.17	5.4
Acetaminophene		0.46	9.38
Ethanol		-0.32	15.9
Citric acid		-1.64	2.79
Citric acid		-1.64	2.79

The tolerance limit was taken as the maximum concentration of the foreign substances, which caused an approximately ±5% relative error in the determination. The results after the experiments revealed that neither 300-fold citric acid, uric acid and ethanol nor 150-fold Ibuprofen, cocaine, vitamin C, tramadole and nor 100 fold of acetaminophene were interfered with the determination of morphine and codeine.

Real sample analysis: To assess the method applicability, we run the whole determination procedure on various spiked urine samples by repetition of 3 (n=3). In this regard, the samples were prepared as described in the sample preparation section. The results are listed in table 2. Presented results indicate that the modified electrode retained its efficiency for the simultaneous determination of morphine and codeine in real samples with satisfactory results.

Table: 4. Simultaneous determination of morphine and codeine in urine samples using our proposed sensor

Samples[a]	Morphine				Codeine			
	Added	Founded	RSD[b]	Recovery	Added	Founded	RSD	Recovery
Person 1	-	<LOD	-	-	-	<LOD	-	-
	3.00	2.94±0.09	3.06%	98%	1.50	1.48±0.14	8.30%	98%
	1.00	1.03±0.02	2.01%	103%	5.00	4.97±0.05	1.01%	99%
Person 2	2.30	2.26±0.07	3.19%	98%	2.30	2.36±0.04	2.08%	102%
	7.50	7.36±0.04	0.61%	98%	7.50	7.36±0.26	3.63%	98%
	0.50	0.54±0.1	19.6%	108%	0.80	0.87±0.5	6.08%	108%
Person 3	-	<LOD	-	-	3.50	3.53±0.04	1.14%	100%
	-	<LOD	-	-	7.50	7.48±0.03	0.51%	99%
	3.80	3.78±0.05	1.45%	99%	-	<LOD	-	-

a. Samples are collected from 3 healthy males which recently didn't take any type of drug or painkiller.
b. *RSDs are based on 3 measurements (n=3).*

CONCLUSION

In this work we applied calf thymus dsDNA as a SPCE strips modifier to develop a sensitive biosensor for simultaneous determination of morphine and codeine in the urine real samples. Accordingly, we evaluate the effect of the amount of immobilized dsDNA on the CV and DPV oxidation pattern of morphine and codeine. Similar to dsDNA study, the effect of pH on the characteristic of differential pulse voltammograms of the two compounds were analyzed. In the case of obtaining a well optimized configuration we applied the biggest potential distance of morphine and codeine peaks and maximum current of signals. These parameters were controled by adjusting the dsDNA concentration and pH. In the case of improve the LODs and applying a matrix cleanup we coupled the current method with EME technique.

Finally, the proposed electrochemical biosensor was used successfully for simultaneously determination of morphine and codeine in urine real sample with satisfactory results.

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REFERENCES

[1] Raffa R. B. A novel approach to the pharmacology of analgesics. Amer. J. Med. 101: S40-S46.
[2] Ensafi AA, Heydari-Bafrooei E, Rezaei B. Different interaction of codeine and morphine with DNA: A concept for simultaneous determination. Bio. Bioelect. 2013; 41: 627-633.
[3] Michel PE, Fiaccabrino GC, De Rooij NF, Koudelka-Hep M. Integrated sensor for continuous flow electrochemiluminescent measurements of codeine with different ruthenium complexes. Analyt. Chim. Acta. 1999; 392(2–3): 95-103.
[4] Greenway GM, Nelstrop LJ, Port SN. Tris (2,2-bipyridyl) ruthenium (ii) chemiluminescence in a microflow injection system for codeine determination. Analyt. Chim. Acta. 2000; 405(1-2): 43-50.
[5] Gustavsson E, Andersson M, Stephanson N, Beck O. Validation of direct injection electrospray lc-ms/ms for confirmation of opiates in urine drug testing. J. Mass Spect. 2007; 42(7): 881-889.
[6] Meatherall R. Gc-ms confirmation of codeine, morphine, 6-acetylmorphine, hydrocodone, hydromorphone, oxycodone, and oxymorphone in urine. J. Analyt. Toxicol. 1999; 23(3): 177-186.
[7] Lee H-M, Lee C-W. Determination of morphine and codeine in blood and bile by gas chromatography with a derivatization procedure. J. Analyt. Toxicol. 1991; 15(4): 182-187.
[8] Delbeke FT, Debackere M. An international journal on all drug-related topics in pharmaceutical, biomedical and clinical analysisinfluence of hydrolysis procedures on the urinary concentrations of codeine and morphine in relation to doping analysis. J. Pharmac. Biomed. Analy. 1993; 11(4): 339-343.

[9] Gomez MR, Sombra L, Olsina RA, Martínez LD, Silva MF. Development and validation of a capillary electrophoresis method for the determination of codeine, diphenhydramine, ephedrine and noscapine in pharmaceuticals. Il Farmaco. 1993; 60(1): 85-90.
[10] Gottardo R, Bortolotti F, De Paoli G, Pascali JP, Mikšík I, Tagliaro F. Hair analysis for illicit drugs by using capillary zone electrophoresis-electrospray ionization-ion trap mass spectrometry. J. Chromat. 2007; 1159(1-2): 185-189.
[11] Domínguez Vidal A, García Reyes JF, Ortega Barrales P, Molina Díaz A. Uv spectrophotometric flow-through multiparameter sensor for the simultaneous determination of acetaminophen, acetylsalicylic acid, and caffeine. Anal. Lett. 2002; 35(15): 2433-2447.
[12] Porath D, Bezryadin A, De Vries S, Dekker C. Direct measurement of electrical transport through DNA molecules. Nat. 2000; 403(6770): 635-638.
[13] Bixon M, Giese B, Wessely S, Langenbacher T, Michel-Beyerle ME, Jortner J. Long-range charge hopping in DNA. Proceed. Natio. Acad. Sci. 1999; 96(21): 11713-11716.
[14] Wang J, Rivas G, Cai X, Palecek E, Nielsen P, Shiraishi H, Dontha N, Luo D, Parrado C, Chicharro M, Farias PAM, Valera FS, Grant DH, Ozsoz M, Flair MN. DNA electrochemical biosensors for environmental monitoring. A rev. Analy. Chim. Acta. 1997; 347(1-2): 1-8.
[15] Lucarelli F, Kicela A, Palchetti I, Marrazza G, Mascini M. Electrochemical DNA biosensor for analysis of wastewater samples. Bioelect. 2002; 58(1): 113-118.
[16] Marrazza G, Chianella I, Mascini M. Disposable DNA electrochemical biosensors for environmental monitoring. Analy. Chim. Acta. 1999; 387(3): 297-307.
[17] Marrazza G, Chiti G, Mascini M, Anichini M. Detection of human apolipoprotein e genotypes by DNA electrochemical biosensor coupled with pcr. Clin. Chem. 2000; 46(1): 31-37.
[18] Erdem A, Ozsoz M. Electrochemical DNA biosensors based on DNA-drug interactions. Electroanalysis. 2002; 14(14): 965-974.
[19] Fojta M. Electrochemical sensors for DNA interactions and damage. Electroanalysis. 2002; 14(21): 1449-1463.
[20] Ozsoz MS, Electrochemical DNA biosensors. 2012; Pan Stanford.
[21] Holmlin RE, Dandliker PJ, Barton JK. Charge transfer through the DNA base stack. Angew. Chemie Int. Edit. Engl. 1997; 36(24): 2714-2730.
[22] Talemi RP, Mashhadizadeh MH. A novel morphine electrochemical biosensor based on intercalative and electrostatic interaction of morphine with double strand DNA immobilized onto a modified au electrode. Talanta. 2015; 131: 460-466.
[23] Gjelstad A, Rasmussen K, Pedersen-Bjergaard S. Electromembrane extraction of basic drugs from untreated human plasma and whole blood under physiological ph conditions. Anal. Bioanal. Chem. 2009; 393(3): 921-928.
[24] Ahmar H, Tabani H, Hossein Koruni M, Davarani SSH, Fakhari AR. A new platform for sensing urinary morphine based on carrier assisted electromembrane extraction followed by adsorptive stripping voltammetric detection on screen-printed electrode. Bios. Bioelect. 2014; 54: 189-194
[25] Balchen M, Gjelstad A, Rasmussen KE, Pedersen-Bjergaard S. Electrokinetic migration of acidic drugs across a supported liquid membrane. J. Chromat. A. 2007; 1152(1-2): 220-225.
[26] Seidi S, Yamini Y, Rezazadeh M. Electrically enhanced microextraction for highly selective transport of three β-blocker drugs. J. Pharmac. Biomed. Analy. 2011; 56(5): 859-866.
[27] Yamini Y, Pourali A, Seidi S, Rezazadeh M. Electromembrane extraction followed by high performance liquid chromatography: An efficient method for extraction and determination of morphine, oxymorphone, and methylmorphine from urine samples. Analy. Meth. 2014; 6(15): 5554-5565.
[28] Szpakowska I, Krassowska-Swiebocka B, Maciejewska D, Kazmierczak P, Jemielita W, Konrad M, Trykowska J, Maj-Zurawska M. Electrochemical DNA biosensor for testing pentamidine and its analogues as potential chemotherapeutics. Electroanalysis. 2006; 18(13-14): 1422-1430.
[29] Wittmann C, Alegret S, Immobilisation of DNA on chips: Immobilization of DNA on microarrays. 2006; Springer.
[30] Wang J, Cai X, Rivas G, Shiraishi H, Dontha N. Nucleic-acid immobilization, recognition and detection at chronopotentiometric DNA chips. Bios. Bioelect. 1997; 12(7): 587-599.
[31] Wang H, Zhang A, Cui H, Liu D, Liu R. Adsorptive stripping voltammetric determination of erythromycin at a pretreated glassy carbon electrode. Microch. J. 2000; 64(1): 67-71.
[32] Ibrahim MS. Voltammetric studies of the interaction of nogalamycin antitumor drug with DNA. Analy. Chim. Acta. 2001; 443(1): 63-72.
[33] Powell MF. Enhanced stability of codeine sulfate: Effect of ph, buffer, and temperature on the degradation of codeine in aqueous solution. J. Pharm. Sci. 1986; 75(9): 901-903.

[34] Garrido JMPJ, Delerue-Matos C, Borges F, Macedo TRA, Oliveira-Brett AM. Voltammetric oxidation of drugs of abuse ii. Codeine and metabolites. Electroanalysis. 2004; 16(17): 1427-1433.
[35] Carter MT, Rodriguez M, Bard AJ. Voltammetric studies of the interaction of metal chelates with DNA. 2. Tris-chelated complexes of cobalt(iii) and iron(ii) with 1,10-phenanthroline and 2,2'-bipyridine. J. Amer. Chem. Soci. 1989; 111(24): 8901-8911.
[36] Middelthon-Bruer TM, Gjelstad A, Rasmussen KE, Pedersen-Bjergaard S. Parameters affecting electro membrane extraction of basic drugs. J. Separ. Sci. 2008; 31(4): 753-759.
[37] Yazdi AS, Es'haghi Z. Liquid–liquid–liquid phase microextraction of aromatic amines in water using crown ethers by high-performance liquid chromatography with monolithic column. Talanta. 2005; 66(3): 664-669.
[38] Esrafili A, Yamini Y, Ghambarian M, Moradi M. Dynamic three-phase hollow fiber microextraction based on two immiscible organic solvents with automated movement of the acceptor phase. J. Separ. Sci. 2011; 34(1): 98-106.
[39] Salimi A, Hallaj R, Khayatian G-R. Amperometric detection of morphine at preheated glassy carbon electrode modified with multiwall carbon nanotubes. Electroanalysis. 2005; 17(10): 873-879.
[40] Ho K-C, Yeh W-M, Tung T-S, Liao J-Y. Amperometric detection of morphine based on poly (3,4-ethylenedioxythiophene) immobilized molecularly imprinted polymer particles prepared by precipitation polymerization. Analy. Chim. Acta. 2005; 542(1): 90-96.
[41] Ensafi AA, Ahmadi N, Rezaei B, Abarghoui MM. A new electrochemical sensor for the simultaneous determination of acetaminophen and codeine based on porous silicon/palladium nanostructure. Talanta. 2015; 134: 745-753.
[42] Švorc Ľ, Sochr J, Svítková J, Rievaj M, Bustin D. Rapid and sensitive electrochemical determination of codeine in pharmaceutical formulations and human urine using a boron-doped diamond film electrode. Electrochim. Acta. 2013; 87: 503-510.
[43] Pournaghi-Azar MH, Saadatirad A. Simultaneous voltammetric and amperometric determination of morphine and codeine using a chemically modified-palladized aluminum electrode. J. Electro. Chemi. 2008; 624(1-2): 293-298.
[44] Pournaghi-Azar MH, Saadatirad A. Simultaneous determination of paracetamol, ascorbic acid and codeine by differential pulse voltammetry on the aluminum electrode modified by thin layer of palladium. Electroanalysis. 2010; 22(14): 1592-1598.