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Voltammetric studies on the HIV-1 inhibitory drug Efavirenz: The interaction between dsDNA and drug using electrochemical DNA biosensor and adsorptive stripping voltammetric determination on disposable pencil graphite electrode

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ABSTRACT

The interaction of Efavirenz (EFV) with fish sperm dsDNA immobilized onto pencil graphite electrode (PGE) has been studied by using differential pulse voltammetric technique using an electrochemical DNA biosensor. The guanine signal was lower with (double stranded-DNA) dsDNA-treated PGE than the untreated electrode after the interaction with EFV occurred. The changes in the experimental parameters such as the accumulation time and the concentration of EFV were also studied. All necessary parameters such as sensitivity, selectivity, accuracy and precision were calculated. In addition, the detection and determination limits, reproducibility and applicability of the analysis to pharmaceutical dosage forms were also investigated. These results showed that this DNA biosensor could be used for the sensitive, rapid simple and cost effective detection and determination of EFV–dsDNA interaction. The linearity was between 2 and 24 ppm of EFV concentration on guanine signal decreasing curve.

EFV showed an irreversible oxidation behavior at all investigated pH values. This oxidation step was adsorption controlled on PGE. Hence, differential pulse adsorptive stripping (AdsDPV) voltammetric method was developed for the determination of EFV. Accumulation time and potential were optimized. Under these conditions, the current showed a linear dependence with concentration in the range between 0.018 and 2.56 ppm. Both determination methods were fully validated and applied for the analysis of EFV pharmaceutical dosage form.

1. Introduction

DNA recognition layers can be combined with electrochemical transducers to form new and important types of biosensors. DNA offers a powerful tool in the recognition and monitoring of many important compounds. (Mascini et al., 2001; Palecek et al., 2005; Wang, 2006; Blum and Coulet, 1991; Rauf et al., 2005; Marrazza et al., 1999). DNA layers combined with electrochemical transducers produce a new kind of affinity biosensor. The interaction of dsDNA with other molecules especially antiviral and chemotherapeutic drug compounds is an important issue in life sciences. The investigation based on DNA interactions has great importance in understanding the mechanism of action of many drug compounds, designing of new DNA–drug biosensors, and screening of the drugs *in vitro*. Electrochemical DNA biosensors enable the study of the interaction of DNA immobilized on the electrode surface with analytes in solution. Especially, (double stranded-DNA) dsDNA-modified electrodes can be designed for detecting small molecules such as drugs and carcinogens interacting with the immobilized nucleic acid layer. Electrochemical investigations of nucleic acid binding molecules–DNA interactions can provide a useful complement to the spectroscopic techniques and yield information about the mechanism at intercalation and the confirmation of anti HIV–DNA adduct. The immobilization of DNA onto an electrode surface is the crucial aspect in many ways of the developing DNA biosensors for monitoring drugs because it dictates the accessibility of the DNA to drugs in solution and hence can influence the affinity of drug binding. Binding of drugs to DNA and a general DNA damage has been described through the variation of the electrochemical signal of guanine (Jelen et al., 1997a; Jelen et al., 1997b) or adenine (Erdem et al., 2005). Observing the electrochemical signal related to DNA–drug interaction can provide evidence for the interaction mechanism and the nature of the complex formed, binding constant, size of binding site and the role of free radicals generated during interaction in drug action. A good sensitivity of oxidation signals for DNA structure would make carbon electrodes extensively useful in DNA research especially pencil graphite electrode (PGE). Most of the DNA–drug interaction studies are related with

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the anticancer drugs (Brett et al., 2002; Meric et al., 2002; Brett et al., 2006). However, many antiviral drugs, especially anti HIV drugs, are known to exert their biological activities by interacting with DNA.

Efavirenz (EFV) is a human immunodeficiency virus type 1 (HIV-1) specific, non-nucleoside, reverse transcriptase inhibitor (RTI). HIV-1–RTI is required for viral replication, which converts a single-stranded RNA into a double-stranded DNA via polymerase and RNase H activities (Gotte et al., 1999; Sweetman, 2007; Sanbar, 2007). The DNA is then integrated into the host cell genome by HIV integrase. HIV-1 life cycle is the required part of these steps. Given the essential role that RT plays in the life cycle of HIV-1, inhibition of RT has been one of the primary therapeutic strategies in suppressing the replication of HIV-1 (Gotte et al., 1999; Sweetman, 2007; Sanbar, 2007)..

EFV has been studied and determined by other techniques: HPLC for the determination of EFV in plasma (Langmann et al., 2001; Sarasa Nacenta et al., 2001; Matthews et al., 2002; Veldkamp et al., 1999) and in raw S-enantiomer material for the assay of R-enantiomer as impurity (Seshachalam et al., 2008) and spectrophotometric method (Sankar et al., 2003). The reported HPLC methods were influenced by the interference of endogenous substances and potential loss of drugs in the re-extraction procedure and involving lengthy, tedious and time-consuming plasma sample preparation and extraction processes and requiring a sophisticated and expensive instrumentation. There is only one analysis method for the determination of EFV in dosage form with spectrophotometric technique (Sankar et al., 2003). This method is not sufficiently as sensitive like electroanalytical methods.

Electrochemical techniques also help for the identification of the redox mechanism of drug compounds and provide important information about DNA–drug interactions (Palecek et al., 2005; Wang, 2006; Dogan-Topal et al., 2008; Uslu and Ozkan, 2004; Kissinger and Heinemann, 1996; La-Scalea et al., 2002; Ozkan et al., 2003, 2004). The advanced techniques in experimental electrochemical enables simple, low cost, and relatively less time-consuming drug analyses when compared with other techniques.

Conventional DNA biosensors often require chemical or thermal regeneration steps, involving prolonged incubation in hot water, urea or sodium hydroxide solutions (Palecek et al., 2005; Wang, 2006). Electrochemical DNA biosensors, especially using disposable pencil graphite electrode, are assayed more easily and rapidly compared to the conventional DNA biosensors. These single use DNA biosensors have several advantages, such as avoidance of contamination among samples, ease of use because no pretreatment is needed and constant sensitivity and reproducibility (Mascini et al., 2001; Palecek et al., 2005; Wang, 2006).

Electrochemical detection of the interaction between EFV and DNA based on the changes of guanine signal has not been studied on any electrode before. Furthermore, there appears to be no sensitive analytical method for the determination of EFV, either in bulk form or pharmaceutical dosage forms that have been reported. This study is the first electrochemical data of the electrode behavior and the dsDNA–drug interaction of EFV.

In this study, the voltammetric behavior of EFV at a pencil graphite electrode and at a DNA biosensor is presented. The aim of this study was to establish the experimental conditions such as EFV concentration, its interaction time with dsDNA, dsDNA concentration, and the effect of the ionic strength. For investigating this interaction, differential pulse voltammetry and guanine oxidation signal monitoring before and after interaction between EFV and dsDNA is used. This work also aimed to develop a new, fully validated, rapid, selective and simple voltammetric method for the direct determination of EFV in raw material and pharmaceutical dosage forms without any time-consuming extraction, evaporation, and separation steps prior to drug assay. Differential pulse voltammetric method was used at bare and dsDNA modified disposable PGE.

2. Experimental

2.1. Apparatus

The electrochemical measurements and the investigation of guanine oxidation signals were carried out using an AUTOLAB-PGSTAT 30 electrochemical analysis system and GPES 4.9 software package (Eco Chemie, The Netherlands). A conventional three-electrode cell with an Ag/AgCl (BAS; 3 M KCl) reference and a Pt wire counter and the pencil graphite electrode as the working electrodes were used. A standard one-compartment three-electrode cell of 5 mL capacity was used in all experiments. The renewable pencil graphite electrode (PGE, Tombo Japan) that was adopted in the study by Wang et al. (2000) was used in all experiments. A Rotring® Pencil Model Tikky II (Germany) was used as a holder for the graphite lead. Electrical contact with the lead was obtained by soldering a metallic wire to the metallic part. The pencil was held vertically with 15 mm of the lead extruded outside (13 mm of which was immersed in the solution).

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

Operating conditions for DPV studies were: 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 with using AUTOLAB-PGSTAT 30 system. The accumulation potential (700 mV) for the electroanalytical studies was applied for a selected deposit time (300 s) while the solution was stirred at 350 rpm. The other used operating conditions were the same as DPV methods which are given above.

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 (Palecek et al., 2002) using a 'peak width' of 0.01 V.

2.2. Chemicals

dsDNA was purchased from Serva Company (Germany). EFV and its pharmaceutical dosage form were kindly supplied by Merck Sharp & Dohme (Istanbul, TURKEY). All other chemicals were of reagent grade (Merck or Sigma). dsDNA stock solution (10 mg/10 mL) was prepared with ultrapure water and kept at −20 °C. More diluted solution of dsDNA were prepared with 0.5 M acetate buffer (pH 4.80) containing 0.02 M NaCl. The pH study for the electroanalytical determination using bare PGE was performed using Britton–Robinson buffer prepared from a mixture of phosphoric acid, acetic acid and boric acid with NaOH.

Stock solutions of EFV (100 ppm) were prepared in methanol and kept in the dark at 4 °C. EFV working solutions for voltammetric investigations were prepared by dilution of the stock and contained 20% methanol. For the voltammetric studies, Britton–Robinson buffer (0.04 M, pH 2.0–12.0) were prepared in double-distilled water. All experiments were carried out at room temperature. For the linearity and range studies, the calibration equation for DPV technique was constructed by plotting the peak current against EFV concentration using bare PGE and dsDNA modified PGE. The diluted solutions were prepared daily by accurate dilution with the selected supporting electrolyte before use and should protect from light to avoid the degradation. All solutions were kept in the dark

and in a refrigerator. EFV solutions were stable over a one week time.

The ruggedness and precision were checked at different days and results were given as within day and between days precision (as R.S.D.% values) (Riley and Rosanske, 1996; Ermer and Miller, 2005; Swartx and Krull, 1997).

2.3. Disposable electrode preparation for the DNA–drug interaction studies

PGE was pretreated by applying +1.4 V for 60 s in 0.5 M acetate buffer containing 0.02 M NaCl (pH 4.80) without stirring. The dsDNA was immobilized on a pretreated PGE by applying a potential at +0.5 V during 240 s using 350 rpm stirring rate in 2 ppm dsDNA in 0.5 M acetate buffer solution containing 0.02 M NaCl. The electrode was then gently rinsed with acetate buffer (0.5 M at pH 4.8) for 5 s for removal of the unbound dsDNA at the electrode surface. The dsDNA modified PGE was immersed in the blank 0.5 M acetate buffer (pH 4.8) containing 0.02 M NaCl and the oxidation signals of guanine were recorded using DPV method. The procedure was repeated using a new PGE. After immobilization of dsDNA and rinse, the dsDNA modified PGE immersed into 0.5 M acetate buffer solution containing 0.02 M NaCl (pH 4.80) having different concentrations of EFV with 350 rpm stirring for 180 s at open circuit system. The electrode was then rinsed with 0.05 M acetate buffer for 5 s. The oxidation signals of guanine were taken using DPV mode in the blank 0.5 M acetate buffer (pH 4.8) containing 0.2 M NaCl.

2.4. Adsorptive stripping voltammetric assay

AdsDPV analyses were carried out in Britton–Robinson buffer at pH 3.0 with 20% methanol on bare PGE. The accumulation potential (usually open circuit condition) was applied for a selected deposite time (300 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 are as described in Section 2.1.

2.5. Film-coated tablet dosage form assay procedure

Ten Stocrin® film-coated tablets (each tablet contains 600 mg EFV, TiO_2 (E171), FeO (E172), hypromellose (E464), carminic acid (E120) and indigocarmine) were weighed and finely powdered. A portion of this powder, equivalent to 10 mg of EFV was transferred into a calibrated flask and completed to 10 mL with methanol. The content of the flask were sonicated for 10 min for complete dissolution. After the settling of non-dissolved particles, a sample from the clear supernatant was withdrawn and quantitatively diluted with the selected supporting electrolytes for bare PGE or dsDNA modified PGE. This solution was then transferred to a voltammetric cell and DPV were recorded. The nominal content of the tablet amounts were calculated from the corresponding regression equations of previously plotted calibration plots obtained using bare PGE or dsDNA modified PGE.

Potential effects of a possible interference of the matrix components of film-coated tablets were also investigated. Recovery of the analyte of interest from a given matrix can be used as a measure of the accuracy or the bias of the method. In this case, recovery experiments are performed in the presence of the real matrix (Riley and Rosanske, 1996; Ermer and Miller, 2005; Swartx and Krull, 1997) for obtaining the accuracy, reproducibility and checking the interference from the excipients used in the formulations. After addition of pure EFV into the pre-analyzed tablet dosage form, DPV were recorded. After five repeated experiments, the recovery results were obtained.

3. Results and discussion

3.1. Interaction of EFV with dsDNA at PGE surface

Some parts of this procedure were adopted from similar steps in the study by Karadeniz and co-workers (Ozkan et al., 2004; Karadeniz et al., 2007). The redox activity of EFV has not yet been studied. A DP voltammogram obtained in 0.5 M acetate buffer solutions at pH 4.8 containing 0.02 M NaCl after free adsorption during 180 s in a 2 ppm EFV solution is shown in Fig. 1A (long dashed line). The oxidation peak of EFV occurs at about +1.25 V, corresponding to the oxidation of the nitrogen atom on the benzoxazine ring. The electrochemical response of EFV was investigated using bare and dsDNA attached PGE. The changes of the peak currents of EFV with bare and dsDNA attached PGE using DPV technique is shown in Fig. 1A.

First of all, for finding the precision and reproducibility of the dsDNA signal with the proposed method, the signal of 2 ppm concentration level of fish sperm dsDNA was measured. The within day reproducibility results (R.S.D.%) for guanine potential and current were 0.45% and 0.98%, respectively. Between day reproducibility results for peak potential and peak current were 0.46% and 1.58%, respectively.

The DPV peak currents of guanine and EFV were measured before and after the interaction (Fig. 1A). The oxidation peak potential of the guanine and EFV were obtained at +1.0 V and +1.25 V, respectively. The guanine peak obtained with the dsDNA attached PGE was higher than that one obtained after interaction with EFV (Fig. 1A). After the interaction with EFV, there was a linear decrease at guanine signal (Fig. 1B). The EFV signal also decreased after this interaction but this decrease was not linear. A series of three repetitive DPV guanine peak measurements of the interaction of 2 ppm

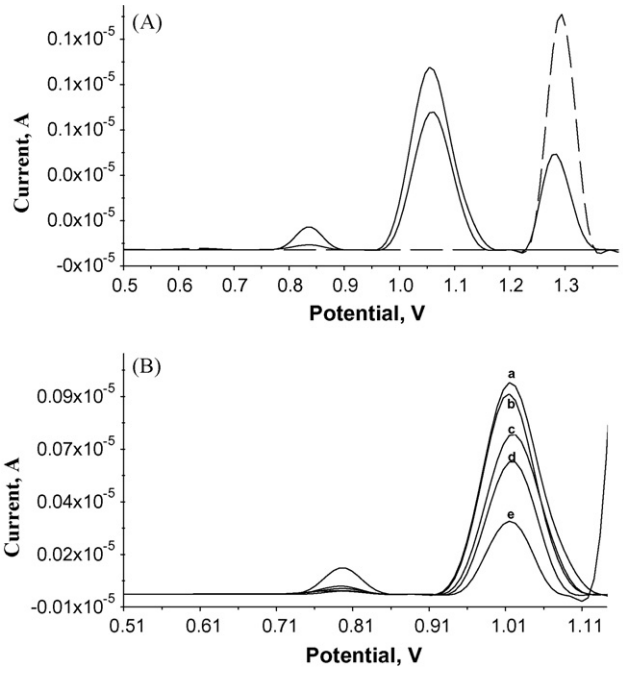

Fig. 1. (A) DP voltammograms for the interaction of 2 ppm EFV with 2 ppm dsDNA at PGE surface; guanine oxidation signal (- - -) before interaction; (—) after interaction of EFV with dsDNA and (– – –) only 2 ppm EFV response (at 0.0 V after 180 s accumulation). (B) DP voltammogram for interaction of 2 ppm dsDNA with different amount of EFV solutions at PGE surface; (a) guanine signal obtained from 2 ppm dsDNA solution; (b) after immobilization of dsDNA on PGE, the obtained guanine signal in acetate buffer solution at pH 4.8 containing 0.02 M NaCl; 2 ppm dsDNA interaction with (c) 2 ppm EFV; (d) 8 ppm EFV; (e) 24 ppm EFV. Experimental conditions as described in Section 2.3.

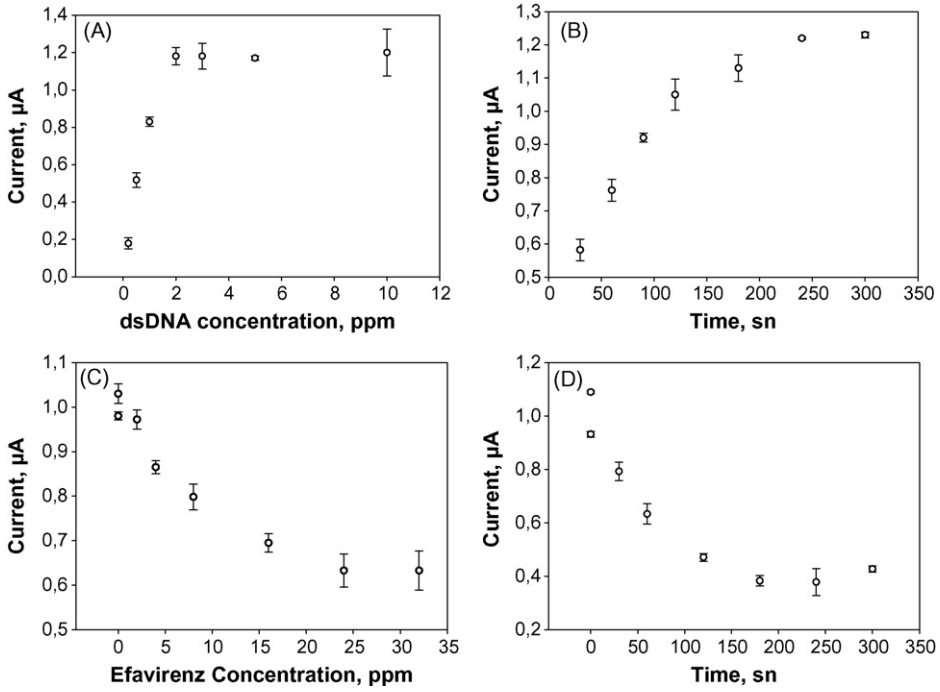

Fig. 2. The effect of dsDNA concentration (A) and time (B) at oxidation signal at PGE for the optimization of immobilization of dsDNA. The effect of EFV at different concentrations (C) and interaction time (D) of EFV with dsDNA on signal of the guanine at dsDNA modified PGE for the optimization of EFV interaction. Experimental conditions as described in Section 2.3.

fish sperm dsDNA with 16 ppm of EFV at PGE resulted in very reproducible results at 2.22% R.S.D. value as between day reproducibility.

The effect of experimental parameters including the effect of dsDNA concentration and accumulation time and the effect of EFV concentration on DNA and its accumulation time were also studied in order to find the optimum analytical performance. For finding the optimum concentration of dsDNA, seven different concentrations between 0.2 and 10 ppm were studied. These studies were realized at +0.5 V using 120 s accumulation time. The other parameters are described in Section 2. The best results were obtained with 2 ppm dsDNA solutions as shown in Fig. 2A. After this step, the accumulation time studied was realized between 30 and 300 s using 2 ppm concentrations. The optimum accumulation time was obtained using 240 s as shown in Fig. 2B.

The effect of experimental parameters including the effect of EFV concentration on dsDNA and EFV accumulation time were also studied in order to find optimum analytical performance for the application to the tablet dosage form. For obtaining the relationship style (linear or nonlinear) between EFV and dsDNA, different EFV concentrations (0–32 ppm) were studied. These studies were realized using 0 V accumulation potential at optimum accumulation time (120 s). The other parameters are described as in Section 2. The best result was obtained at 24 ppm EFV concentration (Fig. 2C). After this step, using 2 ppm dsDNA and 24 ppm EFV concentrations, the optimum accumulation time studies were done between 0 and 300 s. The optimum accumulation time was obtained as 180 s (Fig. 2D).

The 24 ppm concentration of EFV has a pronounced effect its interaction with dsDNA at PGE surface. After interaction with anti-HIV drug of EFV there was a decrease on guanine signal till 180 s (3 min) and then gradually looks constant between 180 and 300 s. Fig. 2C showed that guanine oxidation signal decreased with increasing concentration of EFV up to 24 ppm and then it was not changed worth to investigation. After EFV interaction with dsDNA at PGE surface, the guanine signal of dsDNA decreased. The decrease of the oxidation signal of guanine bases was attributed to the binding of EFV to this electro active DNA base. This situation could be explained by shielding of oxidizable groups of electroactive DNA bases such as guanine while EFV interact with dsDNA at electrode surface or in solution phase. This decrease could be explained as a possible damage in the oxidizable groups of guanine that could cause mutations especially on guanine bases (Erdem et al., 2005; Meric et al., 2002; Ozkan et al., 2004; Palecek et al., 2002; Karadeniz et al., 2007; Wang et al., 1996). PGEs provide simple and rapid detection of the interaction between dsDNA and EFV by non-toxic agents. This kind of experiments are very important to understand of determination for recognition of DNA sites would be valuable in the rational design of new DNA-targeted molecules for application in AIDS or other viruses therapy. Our obtained results showed that PGE might be used for the detection of EFV interaction with dsDNA directly.

3.2. Electroanalytical determination of EFV using dsDNA modified PGE

The results obtained for the EFV oxidation using dsDNA-biosensor demonstrates a good possibility for developing an electroanalytical methodology for EFV detection and determination.

The electroanalytical behavior of EFV is dependent on the electrolyte solutions using dsDNA modified PGE. For this electroanalytical study, 0.5 M acetate buffer at pH 4.8 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 analytical parameters were chosen according to Section 3.1 results. The guanine oxidation peak on dsDNA decreased linearly after interaction with EFV. The decrease in the guanine oxidation peak currents were used as a function of EFV concentrations (at 2, 4, 8, 16 and 24 ppm EFV) to confirm the linear relationship between the current and concentration (Table 1). Adsorptive stripping analysis is an extremely sensitive electrochemical technique for measuring trace amounts of drug compounds and offer well-established advantages including good discrimination against background currents and low detection limits (Wang, 2006; Kissinger and Heinemann, 1996). Specifically,

Table 1
Regression data of the calibration lines for determination of EFV by AdsDPV at dsDNA modified and bare PGE.

	dsDNA modified PGE	Bare PGE
Measured potential (V)	1.02	1.34
Linearity range (ppm)	2–24	0.018–2.56
Number of point	5	9
Slope ($\mu A\,ppm^{-1}$)	−0.0138	2.89
Intercept (μA)	0.704	0.601
Correlation coefficient	−0.999	0.999
SE of slope	0.00029	0.0701
SE of intercept	0.0040	0.069
LOD	0.599	0.0042
LOQ	1.995	0.0138
Within day reproducibility of peak potential (R.S.D.%)	0.29	0.35
Within day reproducibility of peak current (R.S.D.%)	0.96	1.32
Between day reproducibility of peak potential (R.S.D.%)	0.46	0.60
Between day reproducibility of peak current (R.S.D.%)	2.22	1.58

R.S.D.%: relative standard deviation.

adsorptive stripping analysis greatly enhances the scope of stripping measurements toward numerous low amount compounds. In this study, short adsorption time (180 s) result in a very effective interfacial accumulation. In the proposed dsDNA modified PGE method, the peak current is decreased linearly between 2 and 24 ppm concentration range. As an adsorption potential 0 mV was used for EFV adsorption on dsDNA at 180 s. The rapid increase of the current observed at short pre-concentration time is followed by a leveling-off. Hence to stable and linear response, a 180 s interaction time was used for subsequent quantitative determinations with AdsDPV method using at dsDNA modified PGE. The linear decrease in the concentration range between 2 and 24 ppm using at dsDNA modified PGE indicates that the response was adsorption-controlled dsDNA–EFV concentration in this range. Above this concentration (32 ppm), a loss of linearity was observed. The characteristics of this calibration plot are summarized in Table 1. The low values of SE of slope and intercept and good correlation coefficient value (0.999), established the precision of the proposed method. In this study, LOD and LOQ values for EFV with dsDNA modified PGE following the measurement procedure were calculated (Table 1) using the following equations (Riley and Rosanske, 1996; Ermer and Miller, 2005):

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

The abbreviation of s is the standard deviation of the peak current (three runs) of the lowest concentration of the linearity range (2 ppm EFV), m is the slope of the related calibration curve. Both the LOD and LOQ values confirmed the sensitivity of the proposed method (Table 1). The precision of the method 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 of the measuring potential and current (after interaction with dsDNA) were calculated and given in Table 1. The within day and between day precision, accuracy and reproducibility were determined as the R.S.D.% and the results were shown in Table 1.

3.3. Electrochemical investigation of EFV at bare PGE

No previous electrochemical data were available concerning the electrode behavior of EFV in the literature. Therefore, several measurements with cyclic (CV) and DPV techniques were performed at PGE, glassy carbon and boron-doped diamond electrode using Britton–Robinson and acetate buffers at different pH values in order to obtain such information. To demonstrate the usefulness of a solid electrode for determination of EFV, which may offer advantages for the use of such electrodes as sensors, the electrochemical behavior of EFV on a PGE was investigated in this study. In fact, the glassy carbon and boron-doped diamond electrode responses were also investigated. The best and sharp responses were obtained with PGE compared with glassy carbon electrode. Interestingly, no measurable or detectable response was obtained with boron-doped diamond electrode. For this reason, further electroanalytical investigation experiments were realized using PGE. As a first step, EFV was subjected to cyclic voltammetric studies with the aim of characterizing its electrochemical behavior. As the second step, EFV was subjected to a voltammetric determination with the AdsDPV mode. EFV is manifested on current-voltage curves recorded by CV on a PGE by one main anodic peak in all investigated pH values. After pH 10, this peak is totally disappeared. Hence, our pH investigations were realized between pH 1.93 and 10.0. The cyclic voltammograms of the 30 ppm EFV in Britton–Robinson buffer at pH 3.0 at a sweep rate of 100 mV s^{-1} on the PGE showed that an anodic peak appeared at about 1.40 V in the initial anodic scan (S.1A). Upon scan reversal, no corresponding reduction peak was obtained to the anodic peak on the cathodic branch. However, on the cathodic branch at 0.63 V one small reduction peak was appeared. On the second positive sweep, two new small additional anodic peaks appeared at a potential less positive than that of the drug namely Ox_2 and Ox_3 at about 0.70 V and 1.04 V, respectively. These additional anodic peaks only appeared after the initial oxidation of EFV and it may be suggested that some chemical follow-up reaction has occurred at the initial charge transfer Ox_2 and Red_1 form a new reversible redox couple (S.1B).

The peak potential of the oxidation process moved to less positive potentials by raising the pH (until pH 10). The variation of peak potential and current of the main peak (Ox_1) with pH for 2 ppm EFV by DPV and CV was investigated between pH 1.93 and 10 in detail. The plot of the peak potential (E_p) versus pH showed one straight line between pH 1.93 and 10, which can be expressed by the following equation in Britton–Robinson buffer:

$$E_p(\mathrm{mV}) = 1584 - 65.25\mathrm{pH} \quad (r:\ 0.996;\ n = 9)\ \text{for DPV}$$

EFV oxidation signal disappeared after pH 10.0. This point is close to the pK_a value of EFV (http://www.cal-tox.org/Downloads/Monographs/Efavirenz.pdf). This point can be explained by changes in protonation of acid–base functions in the molecule. The influence of pH on the EFV peak current was also studied. The peak current versus pH experiment shows that the peak current is maximum in the acidic media. Therefore, a pH of 3.0 Britton–Robinson buffer was deemed an optimal value and supporting electrolyte for subsequent analysis.

When the scan rate varied from 5 to 100 mV s^{-1} in 30 ppm solution of EFV, a linear dependence of the peak intensity (i_p; μA) upon the scan rate (ν; mV s^{-1}) was found, confirmed an adsorptional behavior. The equation is noted below in Britton–Robinson buffer at pH 3.0:

$$i_p\,(\mu A) = 0.125\nu(\mathrm{mV\,s^{-1}}) + 1.06, \quad r = 0.994\,(n:\ 6)$$

A plot of logarithm of peak current versus logarithm of scan rate gave a straight line with a slope of 0.81, close to the theoretical value of 1.0, which is expressed for an ideal reaction the adsorption controlled electrode process (Laviron et al., 1980).

The equation obtained is:

$$\log i_p\,(\mu A) = 0.81 \log \nu\,(\mathrm{mV\,s^{-1}}) - 0.49, \quad r = 0.997\,(n = 6)$$

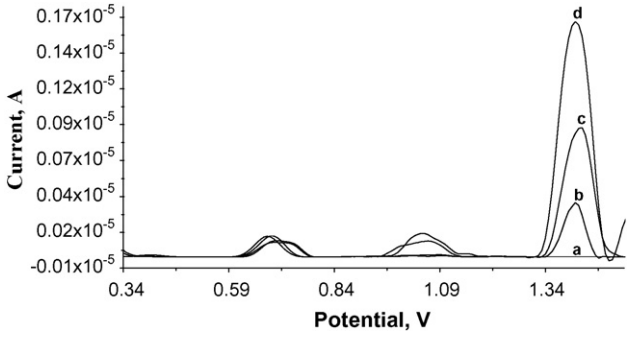

Fig. 3. AdsDPV obtained for the determination in 0.04 M Britton–Robinson buffer at pH 3.0 with a constant amount methanol (20%). (a) Blank; (b) 0.08 ppm EFV; (c) 0.16 ppm EFV and (d) 0.32 ppm EFV.

According to the results obtained from the oxidation peak, the electrochemical reaction was found as adsorption-controlled process. An 80 mV positive shift in peak potential also confirmed the irreversibility of the oxidation process with the absence of the cathodic wave.

3.4. Analytical parameters and validation of the developed AdsDPV method

Adsorptive stripping pulse voltammetric techniques such as AdsDPV are effective and rapid electroanalytical techniques with well-established advantages, including good discrimination against background currents and low detection and determination limits (Wang, 2006; Kissinger and Heinemann, 1996). For realizing this study, all necessary adsorptive stripping parameters such as accumulation time and potential were investigated in Britton–Robinson buffer at pH 3.0 on bare PGE.

The optimum parameters such as accumulation time and potential were investigated as details for 2 ppm EFV. 700 mV as accumulation potential and 300 s as accumulation time was selected and applied for the further studies. A linear relation in the concentration range between 0.018 and 2.56 ppm was found, indicating that the response was adsorption-controlled in this range (Fig. 3). All necessary validation parameters (Riley and Rosanske, 1996; Ermer and Miller, 2005; Swartx and Krull, 1997) such as LOD, LOQ, within day and between day reproducibility, specificity, precision, and accuracy were calculated and listed in Table 1. The low values of SE of slope and intercept and greater than 0.999 correlation coefficient, established the precision of the proposed method. LOD and LOQ were calculated using the described equation in Section 3.2. The precision of the method is also calculated as described in Section 3.2.

Freshly prepared and aged (+4 °C, in the dark) EFV 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.

3.5. Determination of EFV in tablets using dsDNA modified and bare PGE

On the basis of the above results, both dsDNA modified and bare PGE were applied to the direct determination of EFV in tablet dosage forms, using related calibration straight lines without any sample extraction or filtration steps and after an adequate dilutions. These results showed that the proposed methods using dsDNA modified and bare PGE were successfully applied for the assay of EFV in pharmaceutical dosage forms (Table 2). There is no official method reported in any pharmacopoeias so far for the determination of EFV in drug dosage forms. For this reason, the spectrophotometric method (Sankar et al., 2003) was used for comparison. Table 2 compares the results of the analysis of EFV between proposed and literature methods. The amounts of EFV are fairly close to the labeled amounts for all techniques. However, the proposed method is sensitive, selective and more precise than the spectrophotometric assay. The *F*- and Student's *t*-tests were carried out on the data and statistically examined the validity of the obtained results by spectrophotometric and AdsDPV methods at dsDNA modified and bare PGE. At the 95% of the 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 and spectrophotometric methods with regard to accuracy and precision. However, the proposed methods could be successfully applied for EFV assay in tablet dosage form without any interference. To prove the absence of interferences by excipients, recovery studies were also carried out for both electrodes (Table 2). The accuracy of the analysis of both methods was determined by calculating the percentage relative error (Bias%) between the measured mean concentrations and actual concentration. The precision value around the mean value should not exceed 5% of the R.S.D.% (Riley and Rosanske, 1996; Ermer and Miller, 2005; Swartx and Krull, 1997) (Table 2).

Table 2
Results obtained for AdsDPV determination in tablet dosage form using dsDNA modified and bare PGE.

	dsDNA modified PGE	Bare PGE	Spectrophotometric method (Sankar et al., 2003)
Labeled claim (mg)	600.00	600.00	600.00
Amount found[a] (mg)	596.02	600.82	602.36
(R.S.D.%)	1.41	1.68	0.96
Bias%	0.66	−0.14	−0.39
$t_{calculated\ value}$	0.17	0.83	$t_{theoretical} = 2.31$
$F_{calculated\ value}$	0.48	0.41	$F_{theoretical} = 2.60$
Added (mg)	12.00	5.00	–
Found (mg)[a]	12.20	4.85	–
R.S.D.%	2.18	1.12	–
Bias%	−1.67	2.92	–

R.S.D.%: relative standard deviation.
[a] Each value is the mean of five experiments.

4. Conclusion

The proposed electroanalytical method is experimentally convenient, sensitive, selective and rapid so that it requires only small amounts of materials. Utilization of this electrochemical biosensor for interaction between dsDNA and EFV is cost effective and it provides rapid detection. The dsDNA modified PGE was used in combination with DPV to obtain the information about the interaction of EFV with dsDNA, based on the changes at guanine signal. The experimental conditions were optimized by using disposable PGE. This electrode improved the reproducibility and the inexpensive cost when it compared with other electrodes. DNA biosensors also eliminate the need for some difficult analyze techniques (Palecek et al., 2005; Wang, 2006; Blum and Coulet, 1991). This biosensor was also used for the determination of EFV for the first time. Short pre-concentration time permits convenient measurements of low concentrations of EFV. The results have also shown that these studies can play a key role in developing newly produced antiviral or AIDS compounds. The electrochemical DNA biosensor technique has produced experimental evidence for the EFV–DNA interaction and these studies contribute to the understanding of the antiviral activity of the important AIDS drugs. The proposed electrochemical stripping methods using dsDNA modified or bare PGE couple the advantage of extremely low detection limits and suitability for online and *in situ* measurements. Both of the proposed techniques

and their applications represent a good alternative for the detection and determination of dsDNA–EFV interaction and drug amount in pharmaceutical dosage form. The electroanalytical methods represent 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 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.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2008.12.005.

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