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VOLTAMMETRIC DETERMINATION OF ETOPOSIDE BY USING *SEPIOLITE* CLAY MODIFIED ELECTRODE AND ITS INTERACTION WITH DNA

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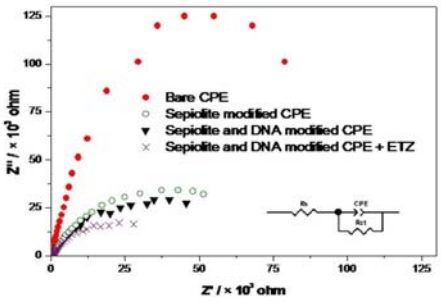
In this study, a carbon paste electrode based on sepiolite clay (CMCPE) as a modifier has been applied to the voltammetric determination of etoposide (ETZ) by using anodic adsorptive stripping voltammetry. Compared with a bare carbon paste electrode (CPE), CMCPE significantly enhanced the sensitivity toward the target analyte.

The experimental conditions optimized for the determination of ETZ in the Square wave adsorptive stripping voltammetric mode (AdsSWV) were as follows: initial potential, 0.50 V vs. Ag/AgCl; final potential, +1.00 V; deposition potential, 0.40 V; deposition time, 30 s, and the scan rate, 100 mV s^{-1}. The developed method offers linearity in the concentration range of 0.01 $\mu molL^{-1}$ – 10 $\mu molL^{-1}$ ETZ with r = 0.9951 and the limit of detection of about 0.00262 $\mu molL^{-1}$.

The interaction of ETZ, an anticancer drug, with calf thymus DNA has been studied by Square wave Voltammetry (SWV) and Electrochemical Impedance Spectroscopy methods (EIS).



INTRODUCTION

Etoposide (4-dimethylepipodophyllotoxin ethylidene-b-D-glucoside) is a potent clinical anticancer agent. It is active against several tumors including small lung cancers, lymphoma, leukemia and Karposi's sarcoma associated with AIDS. It is used as part of the preparatory regimen for bone marrow transplants in patients with advanced hematological malignancies.[1] As a widely used anti-cancer agent has been demanded for research on developing sensitive and rapid analytical methods for monitoring it in quality control and biological samples.[2, 3]

A wide area of analytical methods has already been applied to the analysis of ETZ; most of them based on chromatographic techniques are preferred in the determination of ETZ. However, these methods require highly sophisticated instrumentation and the respective procedures may be time-consuming. Thus, some alternative determination methods can be chosen, such as fluorimetry[4] or voltammetric methods.[1,5] In the voltammetric methods, a large variety of electrodes has been used for increasing sensitivity and selectivity in the monitoring of electrochemical active substances. [6-9]

Carbon based electrodes have been shown applicable as well; namely, the carbon paste electrode (CPE) modified with carbon nanotubes,[10-12] moreover, there were clay (CMCPE) [13-17] modified CPEs employed in the electrochemical stripping analysis mode. By using this electrode, the one-step separation employing specific adsorption of the target organic substance on the electrode surface had been involved, allowing one to analyze voltammetrically the trace level of *anticancer drugs*.[18,19] With respect to ETZ, the applicability of the CMCPE is for the

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first time reported here and the aim of this work is to develop a sensitive and selective square wave anodic adsorptive stripping method for the determination of ETZ by using clay modified carbon paste electrode (CMCPE), which could be utilized for enhancing the sensitivity of the developed method. Moreover, the voltammetric investigation of DNA-drug interactions with modified carbon pastes has contributed to the emergence the mechanism of action of the anti cancer drugs and designing specific DNA-targeted drugs. For this purpose, it has been also investigated to ETZ-calf tymus double stranded DNA interaction by using SWV and EIS.

EXPERIMENTAL

Apparatus

Square wave adsorptive stripping voltammetric (AdsSWV), cyclic voltammetric (CV) and EIS measurements for ETZ were performed with CHI 660C electrochemical workstation. Electrochemical cell has three electrode systems, the working electrode is CPE or CMCPE (MF-2010 and own design), Ag/AgCl reference electrode (CHI 111) and platinum electrode which was used as an auxiliary electrode. pH measurements were carried out with Thermo Orion Model 720A pH ion meter having an Orion combined glass pH electrode (912600) which had been calibrated with pH 4.13 and pH 8.20 stock buffer solutions before measurements. Double-distilled deionized water was supplied from Human Power I^+, Ultra Pure Water System. All measurements were performed at room temperature.

Reagents and solutions

Clay material (sepiolite), graphite powder, paraffin oil, Double-stranded (ds) calf thymus deoxyribonucleic acid (DNA) and etoposide was purchased from Sigma. 1.0×10^{-3} M stock solution of ETZ was prepared by dissolving in methanol and this stock solution was kept in fridge at +4 °C. Assay solutions were prepared by diluting stock solution with Britton Robinson buffer solution (BR). The BR buffer solution was prepared by using usual way[20] and adjusted to the desired pH by using NaOH solution. All other reagents were of analytical quality grade and double distilled water was used for all the experiments. The stock DNA, solution was prepared by dissolving of 1 mg DNA in 1 mL double distilled water and kept in a fridge.

Preparation of working electrode

For the comparative purposes, two different carbon pastes were prepared by intimate hand-mixing of graphite powder and paraffin oil (bare), graphite powder, parafin oil and sepiolite clay (CMCPE). All the pastes homogenized manually using a pestle and mortar were packed into piston-driven Teflon® holders of own design. Bare carbon paste electrode was prepared by hand mixing of 30 mg graphite powder (Fluka, %99, ≤ 20 μm) and 10 μL paraffin oil (Aldrich) in a mortar and pestle for 10 min. To prepare clay modified carbon paste electrode, firstly, optimum proportion of clay and graphite powder was mixed and then 10 μL paraffin oil was added in a mortar and pestle. Both bare and clay modified pastes were packed into the hole of the electrode body and the electric contact was made with a copper wire in the center of the rod. The surface of the paste was polished with a part of paper until it had a shiny appearance.

Analytical procedure

10 mL of BR buffer was added in a voltammetric cell and the pH was adjusted to desired value, then required aliquot of stock solution of ETZ was placed into the cell. For oxygen removal, the solution in the cell was purged with nitrogen gas before the experiment and all the rest of the experiments. After that the working electrode was placed in the cell and ETZ was accumulated on the electrode at optimum accumulation potential and time. The stirring was stopped and after 10 s of rest, the anodic sweep was carried out between 0.5 and 1.0 V.

Voltammetric and impedance analysis of DNA-ETZ interactions

Firstly, DNA interaction of ETZ was investigated by using square wave voltammetry technique. The 1.0×10^{-6} M ETZ solution was prepared in voltammetric cell and then the desired volumes of stock DNA solution were added and after each adding, the SWV voltammogram was recorded. Secondly, the stock DNA solution was dropped onto the CMCPE electrode surface and dried in room temperature. Before modification and after modification of electrode surface, the impedance measurements were recorded in BR. Then, the ETZ was added into the solution and again the impedance measurement of electrode surface was recorded. The frequency range at the impedance spectroscopy was 0.1-10^5 Hz. All experiments were carried out at 25°C.

RESULTS AND DISCUSSION

Cyclic voltammetry

First of all, two different electrodes were compared for their suitability in the role of sepiolite clay modifier. The electrodes were tested by using cyclic voltammetry with of 1.0×10^{-4} M ETZ in BR buffer solution pH 2.0 (see Fig. 1). Electrochemical response of ETZ gave rise to a well-defined anodic peak at potentials near to 0.6 V vs. ref. within the first scan, the backward scanning then not resulted in cathodic peaks. As seen in the Fig. 1 a, b, the corresponding CV has exhibited the highest peak currents (compared to the bare CPE). The oxidation peak of ETZ at CMCPE is negationed more negatively than at bare CPE, which affect the determination of ETZ as electrocatalylitic effect.

To optimize the amount of Sepiolite clay in the carbon paste mixture, six types of CMCPE were prepared containing 3, 7, 10, 13, 17 and 20% (w/w) of the modifier. The respective CV curves,

i.e. measurements already directly associated with the optimization of the analytical procedure, obtained in the same solution of ETZ (with 1.0×10^{-4} mol L^{-1}) at pH 2.0 have proved clearly that the optimum signal-to-noise characteristics, peak current intensity, as well as the proper consistency and good mechanical properties, were obtained with the CMCPE containing 7% ETZ (not shown). This paste was thus chosen for further measurements.

The influence of scan rate on peak potential and peak current was investigated for determining electrode process of ETZ. Scan rate changed in the range of 0.05-1.0 Vs^{-1} and seen that the peak poetntial shifts to more anodic values with increasing scan rate. This behavior indicates irreversible nature of oxidation process. When the scan rate varried from 0.05-1.0 Vs^{-1} in 1.0×10^{-4} M ETZ solution, plot of logarithm of peak current versus logarithm of scan rate gave a straight line with a slope of nearly 0.60. These result confirm that the electrode process is controlled by adsorption under diffusion condition.[20]

The CV curves obtained in the series of Britton-Robinson buffers (with pH between 2.0 and 9.0) had contained the single irreversible oxidation signal. The influence of pH on the peak current of ETZ at CMCPE was given in Fig. 2.

As seen in Fig. 2a, the peak current of ETZ decreased with the increase of pH until the pH value of 5.0. After 5.0, peak current value nearly the same with the increase of pH value. According to these results, the sharpest and most favorably developed peak was obtained at pH 2.0.

pH influences the electrochemical behavior of molecules. By using proton and electron numbers calculated from pH and peak potential values (Fig. 2b), the oxidation mechanism of ETZ is estimated. Proton number which accompanied electrochemical oxidation reaction of ETZ was calculated from the literature equation (1)[21]

$$E_p = E^0 + \frac{RT}{nF}\ln\frac{[Ox]}{[\mathrm{Re}\ d]} - \frac{\partial RT}{nF}\ln[H^+] \quad (1)$$

In this equation, ∂ is the proton number which accompanied the oxidation mechanism of ETZ. By using the slope of peak potential versus pH graph (Fig. 2b) and equation (1), ∂/n ratio was calculated and found 0.47, this result shows that 2 e^- was transferred and H^+ was added to oxidation mechanism. Because of these protons, the peak current of ETZ is decreased with increasing pH values. Also this result confirmed the obtained proton/electron numbers in literature.[1]

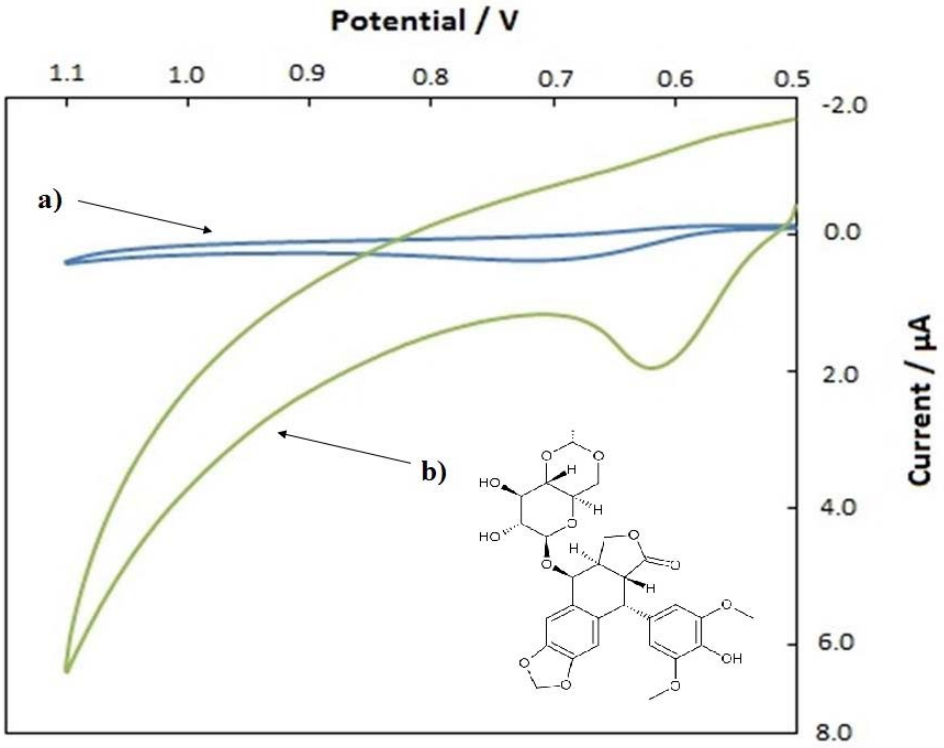

Fig. 1 – Cyclic voltammograms of 1.0×10^{-4} M ETZ **a)** Bare CPE **b**) CMCPE, pH 2.0, scan rate 0.01 V/s.

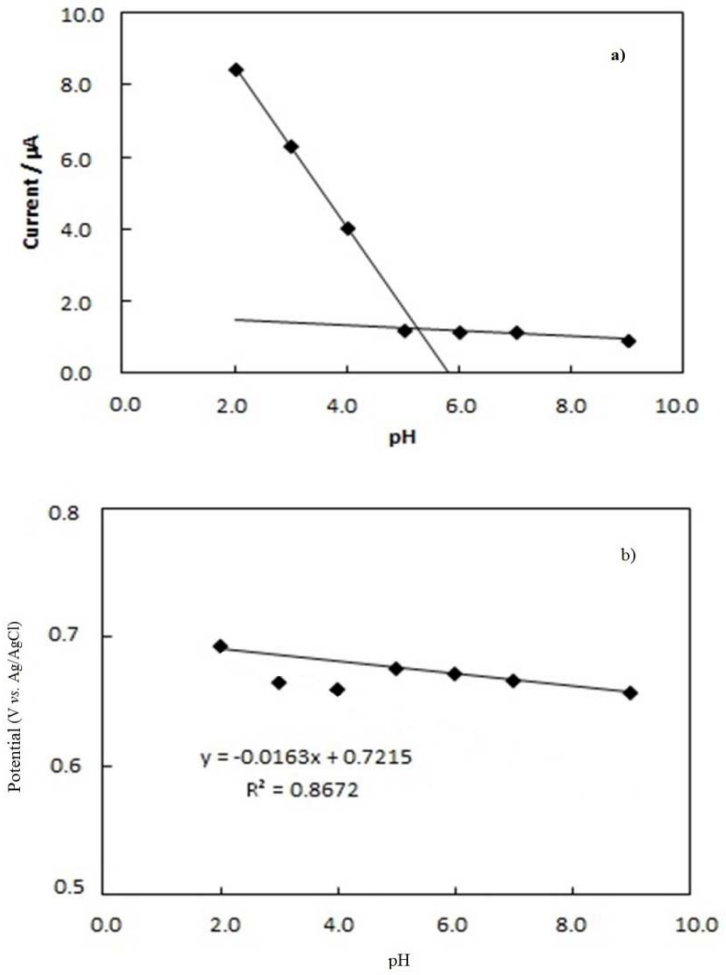

Fig. 2 – Effect of pH on CV responses of 1.0×10^{-4} M ETZ at CMCPE in BR buffer. a) pH – Ip, b) pH – Ep.

Stripping voltammetric studies

In this part of this work, to develop an analytical method, square wave adsorptive stripping voltammetric method (AdsSWV) was used. Also optimum device parameters were determined and the results are given in Table 1.

The current intensity, *I*pa, was found to be dependent upon deposition potential, *E*dep. between 0.20 and 1.0 V vs. ref. As shown in Fig. 3a, the peak current increased to a potential of 0.40 V and then started to decrease. According to the peak shape and peak current, most suitable deposition potential was determined as 0.4 V (Fig. 3a).

Another optimized parameter was the deposition time *t*dep, Fig. 3b illustrates that the oxidation signal has sharply increased to the deposition time to 30 s, whereas the deposition time (up to 30 s) did decrase the peak significantly. As the optimum, a period of 30 s was selected to achieve down to the low level (Fig. 3b).

Table 1

Optimum device parameters for AdsSWV

Parameter	
Initial potential (V)	0.5
Final Potential (V)	1.0
Increament Potential (V)	0.004
Amplitude (V)	0.025
Frequency (Hz)	15
Quiet Time (s)	5

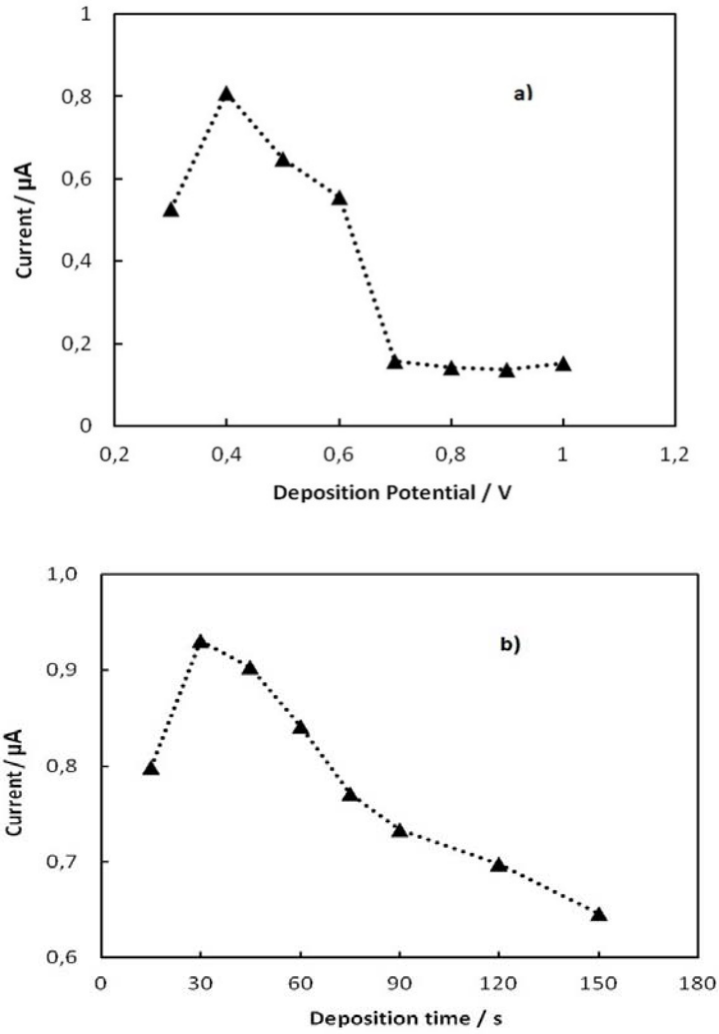

Fig. 3 – **a)** Effect of deposition potential (1.0×10^{-6} M ETZ, pH 2.0, deposition time; 15 s),
b) effect of deposition time on peak current (1.0×10^{-6} M ETZ, pH 2.0, deposition potential; 0.4 V) of ETZ.

By using determinated optimum device and optimum method parameters, calibration study was performed to plot calibration curve. To prove the working concentration range of ETZ different standard solutions were used ranged from 6.67×10^{-9} molL^{-1} to 5.0×10^{-5} molL^{-1}. For each concentration, five reproducible measurements were taken and results of measurements were used to plot the calibration curve. Result of concentration studies indicated that an average peak current of oxidation peak changes linearly with ETZ concentration, in the range from 1.0×10^{-8} molL^{-1} to 1.0×10^{-5} molL^{-1} for AdsSWV. The calibration curve of ETZ can be seen inset in Fig. 4.

The most important stage of developing an analytical method is validation. Validation describes to determine whether the proposed method is suitable for purpose or not. The elements required for method validation are: linearity range, limits of detection and quantitation, accuracy, repeatability, stability, selectivity and robustness.[22]

A calibration curve was composed by using variable ETZ concentrations versus average peak currents. The values obtained from calibration study were evaluated by using the method of least squares and obtained parameters are shown in Table 2.

To indicate the reproducibility of proposed method, the stability of peak currents and peak potentials was investigated. For this purpose, at optimum conditions, ETZ voltammograms were recorded by using AdsSWV and this process was repated 5 times. Relative standard deviation values of peak currents and peak potentials obtained from stripping voltammograms were calculated and found that 1.283 % and 0.349 % respectively. This result shows that the reproducibility of peak potential and peak current values are excellent.

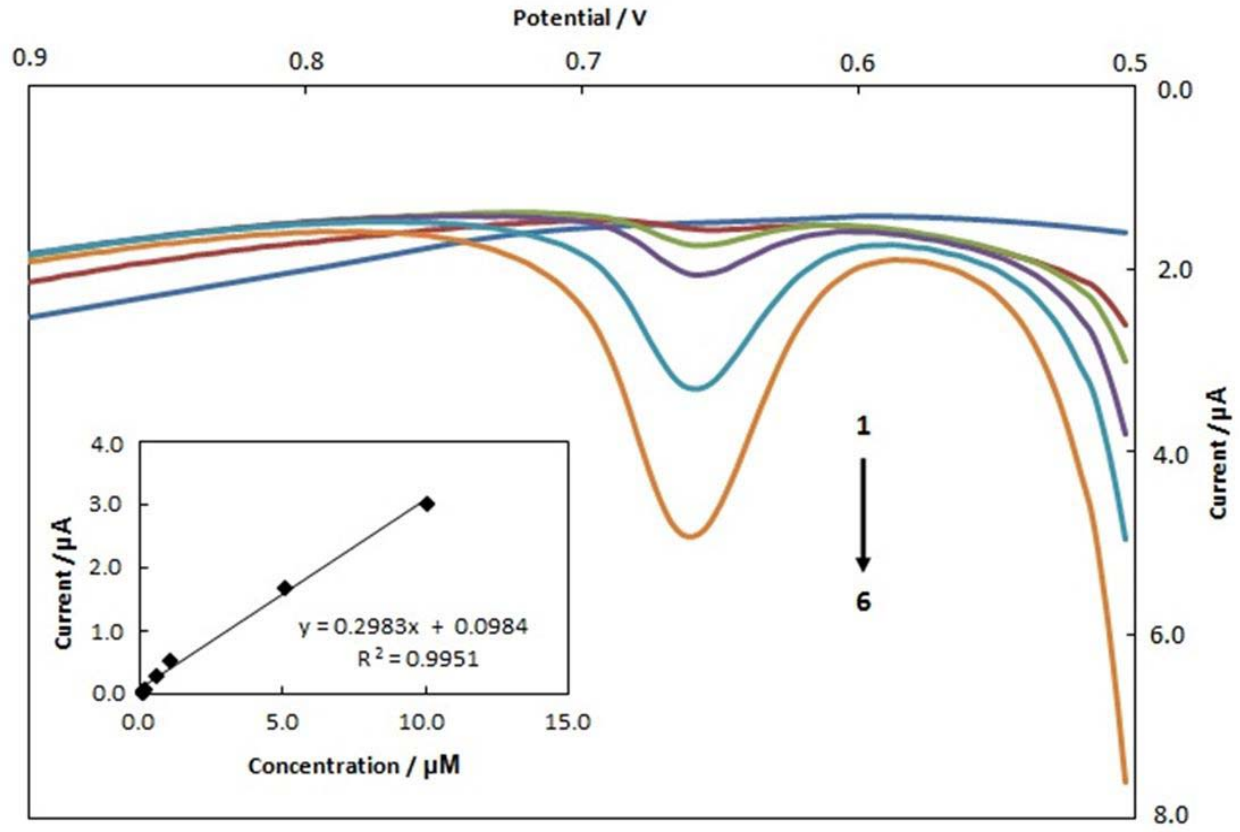

Fig. 4 – The voltamograms of ETZ at different concentrations in AdsSWV (pH:3.0; E_{dep}:0.4 V, t_{dep}: 30 s.). Inset in Fig. 4. Calibration curve of ETZ in AdsSWV. BR (2) 1.0×10^{-7} (3) 5.0×10^{-7} (4) 1.0×10^{-6} (5) 5.0×10^{-6} (6) 1.0×10^{-5} M ETZ.

Table 2

Calibration parameters of developed method

Calibration parameters	**AdsSWV**
Linearity Range, µM	0.01 - 10.00
Potential, V	0.660
Slope of Calibration Curve (m), µA/µM	0.2983
Intercept (b), µA	0.0984
SD of Slope (s_m), µA/µM	0.0094
SD of Intercept (s_b), µA	0.0398
Regression Coefficient (R^2)	0.9951
Limit of Detection (LOD), µM	0.0026
Limit of Quantification (LOQ), µM	0.0087
Repeatability of peak current (RSD %)	1.283
Repeatability of peak potential (RSD %)	0.349

Limit of detection (LOD) and limit of quantitation (LOQ) values were calculated using the relations: LOD = $3s/m$ and LOQ = $10s/m$[23] s is the standard deviation of peak current values of selected concentration of ETZ and m is the slope of the calibration curve. By using this equations, LOD value of AdsSWV is 0.0026 µM. Limit of quantitation (LOQ) value was also calculated by using the specified equation LOQ = $10s/m$, and LOQ value of proposed method was calculated and found 0.0087 µM (Table 2).

DNA interaction studies

The electrochemical oxidation of ETZ was studied in the presence of dsDNA in the BR buffer solution (pH 2.0) on the surface of CMCPE electrode by means of SWV. As seen in Fig. 5, a result of interaction of this drug between the base pairs ds DNA, ETZ peak current decreased.

EIS has been employed to characterize the changes occurring during DNA immobilization at the surface of electrode and DNA-drug interactions.[24]

For this propose, the EIS method was also used and the results are given in Fig. 6, the calculated parameters and obtained fitting electrochemical parameters from using ZView are shown in Table 3. The CPE and CMCPE have the same equivalent circuit recommended in Fig. 6.

The R_{ct} value of bare CPE measured about 11.5×10^5 ohm. This bare CPE electrode modified with sepiolite clay and DNA. Sepiolite modified CPE's R_{ct} value was found to be about 1.2×10^5 ohm. In the presence of DNA on the sepiolite clay modified CPE, the average R_{ct} value was calculated 0.9×10^5 ohm. When DNA + sepiolite modified CPE immersed in 1.0×10^{-4} M Etoposide in BR buffer solution, the R_{ct} value was found to be about 0.53×10^5 ohm.

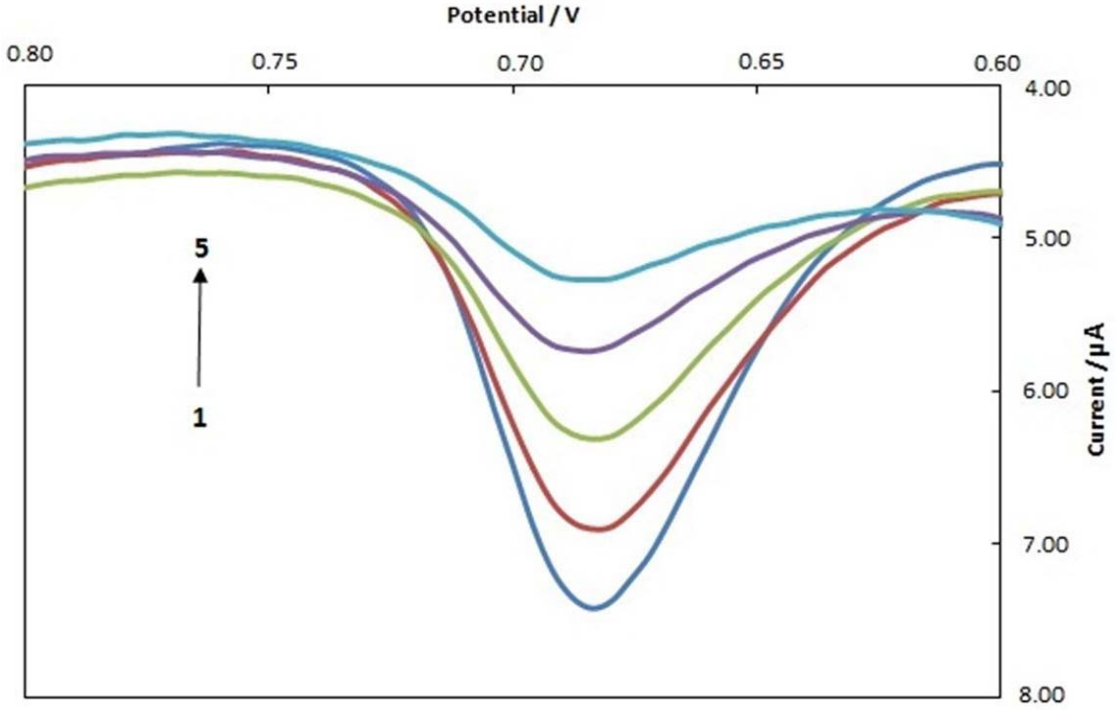

Fig. 5 – SWV voltammograms of 1.0×10^{-6} M ETZ with increasing DNA concentration (pH 2.0, and other parameters are given in Table1) **(1)** 1.0×10^{-6} M ETZ, **(2)** 1.0×10^{-6} M ETZ + 2 ppm DNA, **(3)** 1.0×10^{-6} M ETZ + 4 ppm DNA **(4)** 1.0×10^{-6} M ETZ + 8 ppm DNA **(5)** 1.0×10^{-6} M ETZ + 12 ppm DNA.

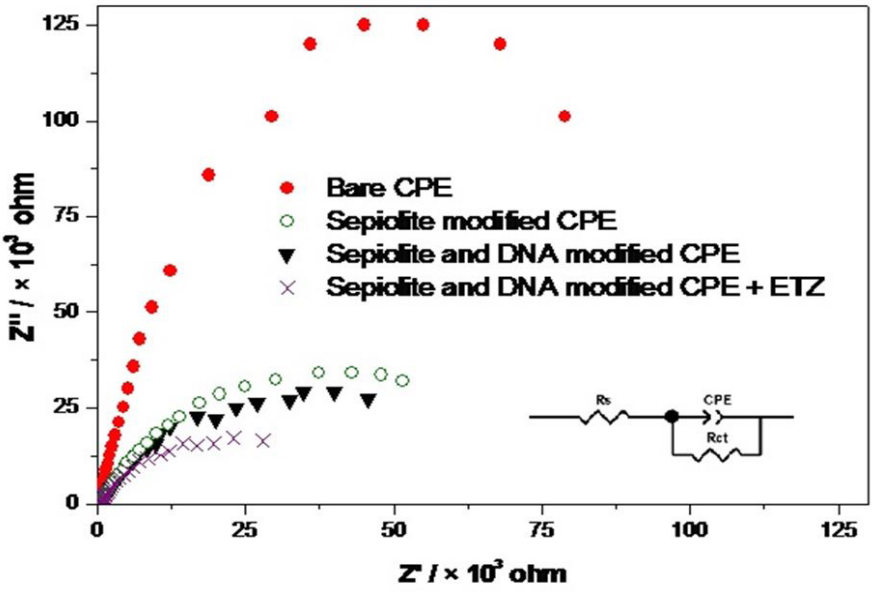

Fig. 6 – Nyquist plots of different electrodes in BR buffer solution at pH 2.0 with and without DNA and ETZ.

Table 3

EIS parameters of CPE and CMCPE in BR buffer solution with and without DNA and ETZ

CPE, Carbon Paste Electrode	**R_s, ohm**	**CPE-T×10^{-5}**	**CPE-P**	**R_{ct}×10^5 ohm**
Bare	243	0.14	0.909	11.50
Sepiolite modified	307	1.33	0.766	1.20
Sepiolite and DNA modified	329	1.51	0.745	0.90
Sepiolite and DNA modified + ETZ in solution	424	2.5	0.730	0.53

DNA molecules increased the surface porosity on sepiolite + DNA electrode. Therefore R_{ct} value decreased from 1.2×10^5 ohm to 0.90×10^5 ohm. With the formation of larger DNA + ETZ molecules increase higher surface porosity of the sepiolite + DNA + ETZ electrode. Similarly R_{ct} value decreased in 1.2×10^5 ohm to 0.53×10^5 ohm. Also, the reactive of ETZ increased with addition of ETZ concentration on sepiolite + ETZ electrode (Fig.4). When addition of DNA concentration in sepiolite + ETZ mixture, free ETZ concentration is decreased in mixture electrode, so that the peak current of ETZ is decreased (Fig. 5). The R_{ct} values are given in Table 3.

According to the results of Nyquist plots, we can say that DNA was modified on the CMCPE surface and when ETZ was added into the solution, DNA- sepiolite modified CPE surface was changed and it can be said that ETZ was in interaction with DNA which was on the surface of the CMCPE. Consequently, the alterations in the EIS response parallel the changes in the voltammetric response.

CONCLUSIONS

In the present work, a sensitive, fast, cost-effective and simple AdsSWV method by using CMCPE was developed for ETZ and due to the CMCPE, as seen in Table 2, LOD and LOQ values showed that the new proposed method is really sensitive. The procedure was simple and precise; it did not require time – consuming extraction or pretreatment steps.

According to our DNA interaction studies, the SWV results demonstrate that ETZ interacts with DNA in solution phase. Furthermore, EIS studies also indicate that DNA interacts with ETZ on the electrode surface.

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