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ELECTROCHEMICAL BEHAVIOR OF THE ANTINEOPLASTIC AGENT ETOPOSIDE AT A GRAPHENE-BASED MODIFIED ELECTRODE: ITS SQUARE-WAVE ADSORPTIVE STRIPPING VOLTAMMETRIC DETERMINATION IN THE PHARMACEUTICAL FORMULATIONS

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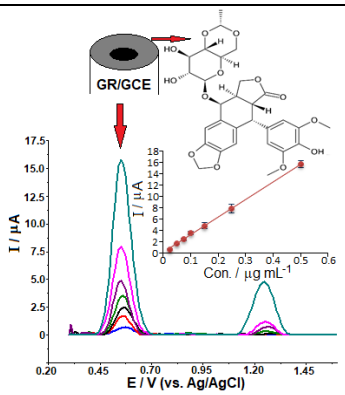
An electrochemical sensor based on the graphene (GR) was prepared, and used for the determination of etoposide (ETO). The electrochemical behaviors of this compound on GR modified glassy carbon electrode (GR/GCE) were investigated by cyclic voltammetry and square-wave adsorptive stripping voltammetry (SWAdSV). Using SWAdSV, a linear calibration curve was obtained for ETO determination in 0.1 M Britton-Robinson (BR, pH 4.0) buffer solution at +0.56 V (vs. Ag/AgCl) (after 30 s accumulation at 0.1 V). A linear relationship was found between peak currents and the concentration of ETO within 0.025 to 0.5 μg mL^{-1}, with a detection limit of 0.0023 μg mL^{-1}. The developed method was successfully applied to the determination of ETO in the pharmaceutical formulations with good recoveries.



INTRODUCTION

Etoposide (4-demethylepipodophyllotoxin ethylidene-D-glucoside) (ETO, Scheme 1) is a drug used to prevent the spread and growth of cancer cells in the body. It is usually given in combination with other cancer medicines to treat a certain type of lung cancer. The mechanism of action of ETO is unknown. However, it is estimated that ETO acts by inhibiting the activity of an enzyme in the cell known as topoisomerase, which is necessary for cell replication and tumor growth. During cleavage procedures, cells need this enzyme to maintain their DNA properly. Blocking the activity of this enzyme breaks down in DNA, which leads to the destruction of cancer cells.[1-3] Previously, the different analytical methods have been developed for the determination of ETO in biological fluids or drug dosage forms including high-performance liquid chromatography with different dedection,[4-12] ultra high-performance liquid chromatography,[13] micellar electrokinetic chromatography,[14-15] and chemiluminescence methods.[16] However, these methods have disadvantages such as taking too much time, needing re-extractions, and using expensive devices and solvents. The electrochemical techniques have the advantage of high precision, accuracy and precision as well as large linear dynamic range and partly less expensive instrumentation. With the development of more precise pulse methods, electrochemical studies have begun to be used more regularly in industry,

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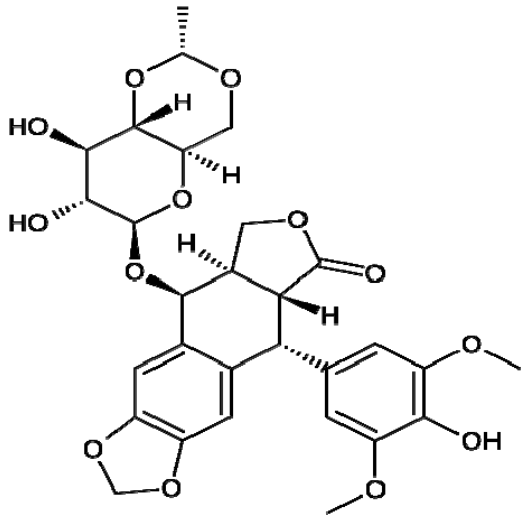
environmental applications and drug analysis, especially in biological samples and tablet forms.[17] Some electrochemical studies on etoposide have been carried out.[18-20] A differential pulse voltammetry method using carbon paste electrode was reported for the determination of ETO in spiked human serum with a detection limit of 0.059 μg mL^{-1}.[18] Bozal-Palabiyik et al.[18] developed an electroanalytical method for the determination of ETO using adsorptive stripping differential pulse voltammetry at a multiwalled carbon nanotube-modified glassy carbon electrode with the detection limit of 0.0032 μg mL^{-1}. In other a study, a carbon paste electrode based on sepiolite clay as a modifier for the voltammetric determination of etoposide by using anodic adsorptive stripping voltammetry was reported with the limit of detection of 0.0015 μg mL^{-1}.[20] The exceptional physical properties like atomic thickness, mechanical strength and charge transport of graphene have placed this material at the forefront of current scientific research. One of the key challenges for the exploitation of this material is to interface it with the molecular world, i.e. to exploit and modify its properties through chemical methods. Modified graphene materials are excellent candidates for electrocatalytic sensing of biomolecules, because of its very large 2-D electrical conductivity, large surface area and low cost.[21]



Scheme 1 – Structure of Etoposide.

In the study, an electrochemical sensor based on the graphene (GR) was prepared and used for the determination of ETO. The electrochemical behaviors of this compound on GR modified glassy carbon electrode (GR/GCE) were investigated by cyclic voltammetry and square-wave adsorptive stripping voltammetry (SWAdSV).

EXPERIMENTAL

Chemicals and solutions

Standard ETO (>99%) was obtained from Biotang Inc. and used without further purification. Analytical grade reagents and purified water from a Millipore Milli-Q system were used for the preparation of Britton-Robinson (BR, 0.1 M, pH 3.0-7.0) buffer solution. Stock solutions of 1.0 mg mL^{-1} ETO was prepared by dissolving in methanol. It was preserved at +4 °C when not in use and protected from daylight during use in the laboratory. Stock solutions were diluted to the desired concentration in selected supporting electrolyte.

Apparatus and measurements

Voltammetric measurements were carried out using an Autolab PGSTAT 128N electrochemical analyzer controlled by GPES 4.9 software package (Eco Chemie, The Netherlands). A three-electrode configuration was composed of a working bare glassy carbon (GCE, 3 mm in diameter, Model MF-2012, BAS) or modified GCE, an Ag/AgCl (3 M NaCl, Model RE-1, BAS) reference electrode and the auxiliary electrode a platinum wire. All electrochemical measurements were carried out in a 10 mL one-compartment electrochemical cell, at a laboratory temperature (20 ± 5 ^{0}C).

Cyclic voltammetry (CV) was employed for preliminary studies on the electrochemical behavior of ETO. Square-wave adsorptive stripping voltammetry (SWAdSV) was used for the development of electroanalytical methodology and the determination of this compound in the pharmaceutical formulations. For stripping voltammetric analysis of samples, BR buffer, pH 4.0 solution was used as the supporting electrolyte. The optimized experimental conditions were as follows: Frequency, 100 Hz; pulse amplitude, 50 mV; scan increment, 10 mV; accumulation time 30 s; accumulation potential, 0.1 V; stirring rate, 600 rpm; resting rate, 5 s. After optimizing the experimental parameters for the proposed method, analytical curves were constructed using modified GCE by adding small volumes of concentrated standard solutions of ETO. All measurements were carried out in triplicate for each concentration.

Preparation of the modified electrode

High-quality graphene (GR) was prepared by an effective chemical method consisting two steps. First, graphene oxide (GO) was synthesized from graphite powder using Hummers′ method.[22] GR was then obtained by reduction of GO according to the literature procedure.[23]

Prior to modification, the bare GCE was polished manually to a mirror-like surface with slurries prepared from 0.05 μm alumina powder on a microcloth polishing pad (BAS). Subsequently, the electrode was rinsed with 1:1 nitric acid-water (v/v), ethanol, and water in an ultrasonic bath for 2 min for each wash, and dried at room temperature. 5.0 mg of GR was added into 2.5 mL of dimethylformamide (DMF), followed by ultrasonication for 2 h to form a homogeneous solution. Next, 10 μL (about 2 mg mL^{-1}) on of the homogeneous dispersion of graphene solution was applied to the clean GCE and allowed to dry in air at room temperature for 8 h. The obtained electrode was noted as GR/GCE. The morphological characterization of the surface of modified electrode was analyzed using scanning electronic microscopy. We have reported the synthesis procedure of GR and characterization of the electrode surface in our previous

work.[24] Prior to use, the GR/GCE was pretreated in the selected buffer solution by cyclic scans between potentials of +0.25 and +1.60 V (10 cycles) at scan rate of 100 mV s^{-1}.

Sample preparation for assay

Aliquots 50 µL of the ETO drug including injectable (Etopex 20 mg mL^{-1}, Deva ilaç Co., Turkey) solution was directly transferred into a 100 mL volumetric flask, followed by making up to volume with methanol. An adequate volume (50 µL) of the resulting solutions were transferred to a voltammetric cell already containing 10 mL of the selected supporting electrolyte (BR buffer pH 4.0) and and analyzed in the day of preparation according to the procedure developed for the pure electrolyte using the calibration curve method from the related regression equation.

RESULTS AND DISCUSSION

The voltammetric behavior of ETO was examined in BR buffer pH 4.0 at the unmodified GCE (curve a, blue line) and GR/GCE (curve b, red line) by cyclic voltammetry in the potential range of 0.25 V-1.6 V (vs. Ag/AgCl) (Fig. 1A). The electrochemical oxidation of 100 µg mL^{-1} ETO under the experimental condition both at the GCE and at the GR/GCE proceeds in two voltammetric oxidation steps (Fig. 1A). The first voltammetric oxidation step (peak i_a) is a irreversible 1 e^- transfer resulting in a stable free radical. The second step (peak ii_a) is an irreversible process, corresponds to the transfer of the second electron. The product formed after 2 e^- oxidation is an unstable cation which undergoes rapid conversion into the o-quinone.[25,26] On the GCE, two oxidation peaks located at around +0.69 V (i_a) and 1.34 V (ii_a), could be observed (Fig. 1A), respectively. Under the same experimental conditions, the oxidation peak currents of ETO obtained at GR/GCE were enhanced and the corresponding oxidation peak potentials shifted to lower positive potentials (+0.59 V for i_a and +1.26 V for ii_a). These results suggesting that the composite provided the best sensitivity, indicating a synergetic activity. The major reason for this improved electrochemical response might be due to the high surface area of the modified electrode on which the electron transfer rate was favored. Also, the good electrical conductivity of the GR can play a crucial role in facilitating the oxidation reaction for ETO.

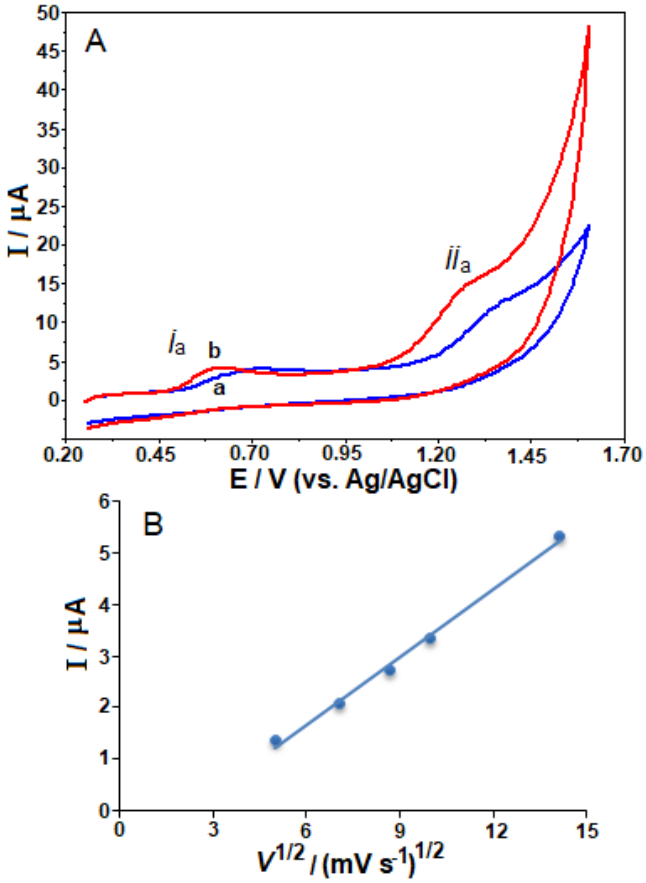


Fig. 1 – A) Cyclic voltammograms of bare GCE (a, blue line) and GR/GCE (b, red line) in Britton-Robinson buffer (pH 4.0) solution containing 100 µg mL^{-1} ETO. Scan rate: 100 mV s^{-1}. B) The plots of the peak current versus square root of scan rate.

The scan rates (v) affect on the voltammetric response of ETO oxidation was investigated by CV at the surface of GR/GCE (data not shown) in BR buffer solution pH 4.0 (most suitable media for analytical purposes). It was found that i_a increases continuously with the scan rate increasing. A linear relationship was found between i_a and the square root of the scan rate ($v^{1/2}$) in the scan range of 25–200 mV s^{-1} (Fig. 1B). The obtained regression equations was i_a (μA) = 0.440 $v^{1/2}$ (mV s^{-1})$^{1/2}$ - 0.977 (n = 5, r = 0.998) for 100 μg mL^{-1} of ETO. Furthermore, the plot of logarithm of the peak current (logi_a) versus the logarithm of scan rate (log v) gave a straight line with a slope of 0.661. The obtained relationship was expressed as: logi_a (μA) = 0.661 log$v$ (mV s^{-1}) – 0.798 (n = 5, r = 0.999). It is well known that the slope of 0.5 for a diffusion-controlled process and a slope of 1.0 for an adsorption controlled process. These equations and the slope value (0.661) indicate that the oxidation of ETO on GR/GCE is mainly controlled by diffusion with some part of adsorption contribution in the selected scan rate range.

After the results given in previous section, further work was dedicated towards analyzing the effect of pH and stripping conditions on the oxidation process of this compound by application GR/GCE. The SWV technique provides higher sensitivity and better peak resolution compared to CV for studying the electrochemical behavior of drug molecules. Thus, this technique was used further experiments. The effect of solution pH on the peak current (i_a) of ETO was studied in the pH range 3.0-7.0 of Britton-Robinson buffer (Fig. 2) by carrying out adsorptive measurements on 1 μg mL^{-1} ETO solution, with an open-circuit mode at 30 s. As can be seen from the Figure 2, when the pH value increased, peak potentials of the compound shifted towards less positive values. The relationship between the anodic peak potential (peak i_a) and the solution pH value could be fit to the linear regression equation of E_p (V) = -0.048pH + 0.743, with a correlation coefficient of r = 0.994. This relationship indicated that protons and electrons take part in the electrode reaction of ETO. In the case of the other anodic peak potential (peak ii_a), it can be concluded that the peak potential practically got unchanged at different pH values. Comparison of two peak currents (i_a, ii_a) of ETO shows that the highest peak current were observed the i_a, so it was subsequently used for the next voltammetric measurements in this work. Meanwhile, the evolution of peak (i_a) currents with pH shows that the highest peak current were observed in pH 4.0. Thus, pH 4.0 BR buffer was considered suitable for the detection of ETO.

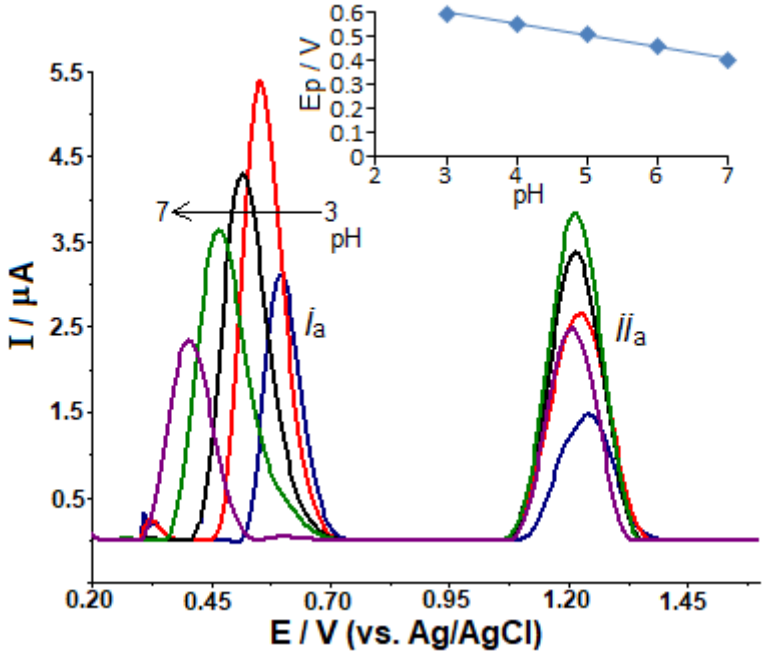


Fig. 2 – The SW stripping voltammograms of 1 μg mL^{-1} ETO solutions in Britton-Robinson buffer pH 3-7 at GR/GCE. t_{acc}= 30 s at open circuit condition; SWV parameters: frequency, 50 Hz; scan increment, 8 mV; pulse amplitude, 30 mV.

Taking into account the pronounced adsorptive characteristics of ETO proved on the GR/GCE, the attention was then turned to the effect of accumulation time (t_{acc}) and accumulation potential (E_{acc}) (data not presented) for 1 μg mL^{-1} ETO under the optimum experimental conditions. The influence of the t_{acc} upon the analytical signal was examined in the range 0-240 s at open-circuit condition. The current increased linearly with t_{acc} till 30 s beyond which the peak current was almost constant, which indicated that the accumulation of ETO at the electrode surface nearly reached a saturation state. The t_{acc} of 30 s is very short and doubtlessly advantageous for practical use of this electrode. After fixing the t_{acc} at this value, the dependence of the stripping peak current on the E_{acc} was evaluated either at open-circuit condition or over the potential range +0.1 to 0.4 V. The stripping peak currents of ETO reached their maximum at an E_{acc} of +0.1. Thus, all the SWAdSV experiments were carried out at 0.1 V.

The dependence of SW response on experimental parameters was finally analyzed in order to optimize the experimental set up for ETO determination. While one parameter was varied all others were kept fixed. When the f changed from 10 to 150 Hz (ΔE_s= 8 mV, ΔE_{sw}= 30 mV) the peak current increased linearly, however the background current and noise were also increased at f values higher than 100 Hz. Thus, f= 100 Hz was selected for all subsequent experiments. When the ΔE_s was changed from 4 to 14 mV, and the remaining parameters were constant (f = 100 Hz, ΔE_{sw}= 30 mV), the recorded signal increased until the value of 10 mV followed by slowlier increase from 10 to 14 mV. In addition, at higher values of 10 mV, an increase in ΔE_s resulted in a broading in the voltammograms were observed. The ΔE_s of 10 mV was chosen for next experiments. The influence of ΔE_{sw} was studied in the range from 10 to 60 mV (remaining parameters: ΔE_s= 10 mV, f = 100 Hz). The peak current of ETO rapidly increased until ΔE_{sw}= 50 mV. However, the best peak morphology and sharper one was obtained at 50 mV, since the peak became wider and deformed at higher values of ΔE_{sw}. Hence, ΔE_{sw}=50 mV was chosen for all following experiments. For entire analysis the optimized values were: f, 100 Hz; ΔE_s, 10 mV; and ΔE_{sw}, 50 mV.

The most suitable chemical conditions and instrumental parameters were established to record the analytical curve for ETO in 0.1 mol L^{-1} BR buffer solution using the GR/GCE. For this, aliquots from the ETO stock solution were consecutively added to the electrochemical cell and the SWAdS voltammetric responses were evaluated for each addition. The SWAdSV responses showed (Fig. 3) that the dependence of peak currents on the ETO concentration was linear, in the range of concentration from 0.025 to 0.5 μg mL^{-1}. The SWAdSV responses were recorded with t_{acc}= 30 s, E_{acc}= 0.1, f= 100 Hz, ΔE_s= 10 mV and ΔE_{sw}= 50 mV. The peak current at a potential of +0.56 V increased proportionally with the ETO concentration (Fig. 3, inset) to yield a highly linear calibration plot; i_p (μA) = 31.339 C (μg mL^{-1}) + 0.118 (r = 0.999, n = 7), where i_p is the adsorptive stripping peak current, C the ETO concentration, r the correlation coefficient, and n the number of experiments.

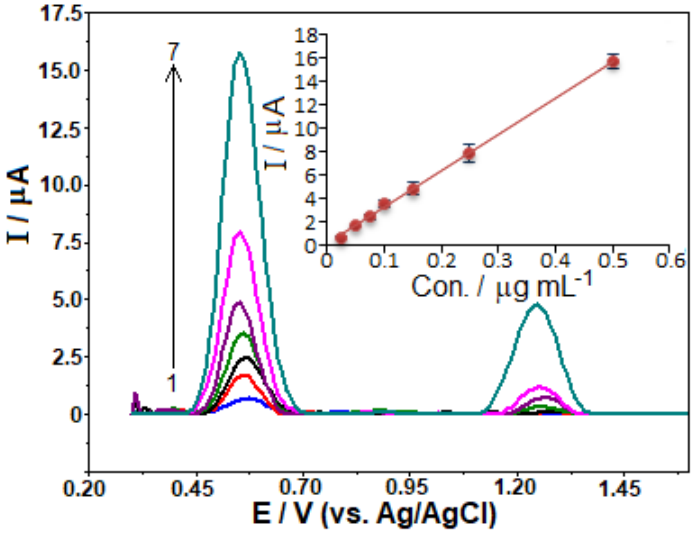


Fig. 3 – The SW stripping voltammograms for ETO levels of (1) 0.025, (2) 0.05, (3) 0.075, (4) 0.1, (5) 0.15, (6) 0.25 and (7) 0.5 μg mL^{-1} in Britton-Robinson buffer (pH 4.0) solution at GR/GCE. Inset depicts a corresponding calibration plot for the quantitation of ETO. t_{acc}= 30 s at 0.1 V; SWV parameters: frequency, 100 Hz; scan increment, 10 mV; pulse amplitude, 50 mV.

Table 1

Comparison of the efficiency of the GR/GCE with literature electrodes for ETO determination

Analyte	Electrode	Linearity range (μg mL^{-1})	Detection Limit (μg mL^{-1})	Reference
ETO	CPE	0.150 – 15.0	0.0590	18
ETO	MWCNT/GCE	0.012 – 1.20	0.0032	19
ETO	CMCPE	0.006 – 5.88	0.0015	20
ETO	GR/GCE	0.025 – 0.5	0.0023	This work

CPE, carbon paste electrode; CMCPE, sepiolite clay carbon paste electrode; ETO, etoposide; GR/GCE, graphene glassy carbon electrode; MWCNT/GCE, multiwalled carbon nanotube glassy carbon electrode.

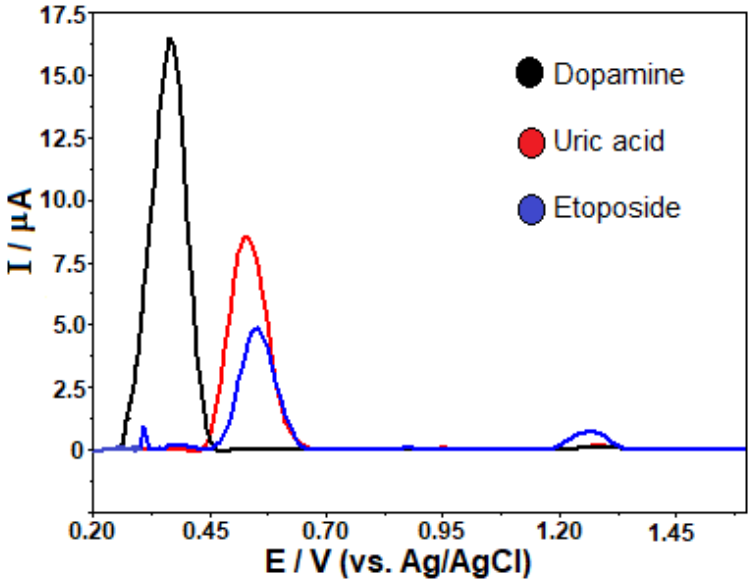


Fig. 4 – The SW stripping voltammograms demonstrating the effect of the presence of various interfering agents on 0.025 μg mL^{-1} ETO (blue line) in Britton-Robinson buffer (pH 4.0). The studied interfering agents were: 0.25 μg mL^{-1} dopamine (black line) and 0.025 μg mL^{-1} uric acid (red line). Other operating conditions as indicated in Fig. 3.

The limit of detection (LOD) and limit of quantification (LOQ) were computed using the formulas 3 *s*/*m* and 10 *s*/*m*, respectively, where *s* is the standard deviation of the peak current (three runs) of the lowest concentration of the related linearity range, and *m* the slope of the related calibration plot. By using these formulas, LOD and LOQ values were found to be 0.0023 μg mL^{-1} and 0.0077 μg mL^{-1}, respectively.

The comparison of the analytical performance of the GR/GCE with other modified electrodes as reported in previous published literatures for ETO determination is given in Table 1. From these data, it can be seen that the analytical parameters obtained by proposed voltammetric method are quite similar [multiwalled carbon nanotube glassy carbon electrode (MWCNT/GCE) and sepiolite clay carbon paste electrode (CMCPE)] or better [carbon paste electrode (CPE)] than those obtained by the others electrochemical methods.

The intra- and inter-day repeatabilities of the GR/GCE were evaluated under the optimum experimental condition. The intra-day repeatability of the magnitude of peak current was determined by successive measurements of 0.025 μg mL^{-1} for ETO solution. The results of ten replicate measurements showed a relative standard deviation (RSD) of 8.10% indicating that the results are repeatable. Further, inter-day repeatability was examined by measuring the magnitude of peak current response of the GR/GCE for three consecutive days for the same concentration of ETO and the RSD was found to be 8.76%. For evaluating the electrode fabrication repeatability, standard ETO solution at the same concentrations was measured by three independent GR/GCEs under the same preparing conditions. The RSD (n=3) values for ETO was calculated as 4.39%, respectively.The experimental results showed that the proposed electrode has a satisfactory repeatability performance for the determination of these compounds. After the modified electrode was stored for 14 days (in the freezer at 4 °C) the stripping peak current of ETO remain to 96.03 of its initial current, which could be attributed to the acceptable storage stability of the proposed electrode.

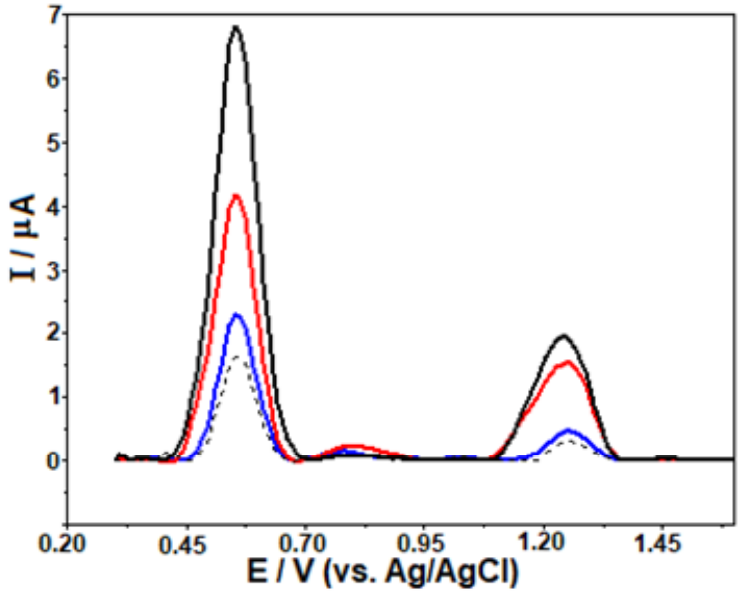


Fig. 5 – The SW stripping voltammograms obtained for the determination of ETO in the spiked sample. Dashed lines represent the diluted sample current. The sample spiked at a ETO levels of 0.025, 0.075 and 0.15. Other operating conditions as indicated in Figure 3.

Table 2

Results of the recovery analysis of ETO in the sample of the drug

ETO added (µg mL^{-1})	Level determined[a](µg mL^{-1})	Recovery (%) ± RSD (%)
-	0.0488	
0.025	0.0729	96.18 ± 7.13
0.075	0.1268	103.96± 5.42
0.15	0.2078	106.02 ± 4.39

[a] Values reported are the average of three independent analysis of each spiked sample

Proposed SWAdS voltammetric method for ETO determination has been tested for the selectivity, by evaluating its response for some common species. The tolerance limit was taken as the concentrations of the foreign substances, which gave an approximately ±5% relative error in the determination of 0.025 µg mL^{-1} ETO. Glucose, fructose, saccharin, sucrose, ascorbic acid, dopamine, uric acid and metal ions such as Na^+, K^+, Ca^{2+}, Mg^{2+} can be present in biological fluids or pharmaceutical samples and may affect on the oxidation of ETO voltammetric responses. The influence of these compounds was checked at the concentration ratios of 1:1, 1:10 and 1:100 in relation to ETO. The obtained results were compared with those obtained for the foreign substance and the foreign substance with the ETO. It was observed that the voltammetric signal of ETO is not influenced by Na^+, K^+, Ca^{2+}, Mg^{2+}, glucose, fructose, saccharin and sucrose even at 100-fold excess and by ascorbic acid, dopamine at up to 10-fold excess (Fig. 4). However, the exposure of same concentration level of uric acid resulted in peak widening because their oxidation peaks overlapped with that of ETO (Fig.4). Based on obtained results it can be concluded that proposed approach provides satisfactory selectivity and can be successfully used for quantification of ETO in pharmaceutical samples analysis.

Next, the applicability of the BDD electrode for SWAdSV determination of ETO was verified by analysis of in the commercially pharmaceutical formulation. The analyzed solutions were prepared as it was described in the sample preparation section, after diluting the resulting solution to a target concentration within the linear range. The dilute real samples were almost similar to aqueous sample in behavior. Quantification for the sample was performed by means of the calibration curve method from the related regression equation. Taking into account the successive dilutions of the sample, ETO content was calculated to be 97.60 mg mL^{-1} (RSD of 6.37%), which approximates the label value of 100 mg mL^{-1} declared by producer. The results achieved by the GR/GCE are in good agreement with the labelled ETO content in the sample, thus indicating the feasibility of the method for ETO determination in pharmaceutical

formulations. The validity was assessed by applying calibration curves and the recovery experiments. Recovery of ETO was calculated by comparing the concentration obtained from the spiked mixtures with those of the pure ETO. Recovery experiments were carried out adding standard ETO solutions (0.025, 0.075 and 0.15 μg mL^{-1}) prepared in supporting electrolyte to 10 mL of sample solution in voltammetric cell and voltammetric responses were evaluated (Fig.5). Recovery of ETO was calculated by comparing the concentration obtained from the spiked mixtures with those of the pure ETO (Table 2). The recoveries ranged from 96.18 to 106.02%, indicating the absence of matrices interference effect in the commercial dosage forms. It was found that ETO amount can be quantitatively recovered by the proposed SWAdSV method, being thus a guarantee of the accuracy of the voltammetric determination of ETO in the commercial pharmaceutical formulation.

CONCLUSIONS

In this work, the GR/GCE was used first time for electroanalytical determination of ETO. The electrochemical behavior of irreversibly oxidized ETO on the GR/GCE was investigated by CV and SWAdSV technique. The obtained results allow concluding that the GR/GCE could be promising alternative with a great potential for the quantitative determination of ETO in pharmaceutical formulations.

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