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Short communication

Investigation of electrochemical behavior of anisomycin on gold electrode followed by HPLC–MS/MS analysis

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ABSTRACT

Anisomycin is an immunosuppressant in low doses (<0.1 μM) with possible application in treatment of some autoimmune diseases and in inhibiting transplantation rejection. Anisomycin suppresses malignant tumor cell growth and affects memory. For the first time it was the subject of the electrochemical investigations by cyclic voltammetry and square wave voltammetry on gold electrode in 0.05 M $NaHCO_3$ using its electrochemical activity. The cyclic voltammetry experiments at different sweep rates show that electrochemical process is irreversible and diffusion controlled. Based on square wave voltammetry measurements, the calculated values of LOD and LOQ were 1 and 4 nM (in the absence of biological fluid), as well as 2 and 6 nM (in the presence of spiked urine) indicating the high sensitivity of the proposed electroanalytical method. High performance liquid chromatography–tandem mass spectrometry was a reference method for quantification of anisomycin and served for structural identification of its hydrolysis product (deacetylanisomycin).

1. Introduction

Anisomycin is a multifunctional antibiotic isolated from two different species of *Streptomyces* [1]. It is also an inhibitor of protein synthesis [2–5] and affects memory by inhibiting the consolidation of new memories and causing amnesia [6–14]. Anisomycin is an immunosuppressant in low doses (<0.1 μM; M = mol l^{-1}) indicating its possible application in treatment of some autoimmune diseases and in inhibition of the transplantation rejection [4,5]. It suppresses malignant tumor cell growth and can be used as an antitumor agent [15–17]. Anisomycin possesses herbicidal activity and selective activity against pathogenic protozoa and fungi [18,19].

This drug has been identified and analyzed using liquid chromatography, ultraviolet and infrared spectroscopy, as well as mass spectrometry [20–22]. To the best of our knowledge, there are no methods reported for the electrochemical determination of anisomycin.

The aim of this work was to investigate the electrochemical activity of anisomycin on gold electrode using cyclic voltammetry (CV) in 0.05 M $NaHCO_3$. Square wave voltammetry (SWV) was applied for quantitative determination of the drug and in spiked urine samples, followed by high performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS). The structural identification of anisomycin and its hydrolysis product in 0.05 M $NaHCO_3$ and under the potential cycling conditions in the absence and in the presence of biological sample (urine) was also performed by HPLC–MS/MS.

2. Experimental part

2.1. Chemicals and reagents

Anisomycin standard (purity ≥ 98%) was obtained from Fermentek (Jerusalem, Israel). Used solvents were HPLC grade from Sigma-Aldrich. Analytical grade trichloroacetic acid (TCA) and $NaHCO_3$ were supplied by Sigma-Aldrich and Merck, respectively. Deionized water was obtained by passing the distilled tap water through a GenPure ultrapure water system (TKA, Niederelbert, Germany).

2.2. Apparatus and preparation of electrode surfaces

Standard equipment, PGZ 402 Volta Lab (Radiometer Analytical, Lyon, France) has been used for the electrochemical measurements and the three electrode electrochemical cell was described in detail previously [23]. Polycrystalline gold (0.07 cm^2) served as the working electrode and a gold wire as the counter electrode. All the potentials are given vs. saturated calomel electrode (SCE) used as the reference one. Preparation and activation of gold electrode was described previously [23]. Electrolyte, 0.05 M $NaHCO_3$ (pH = 8.4) was previously deoxygenated by purging with nitrogen at room temperature. SWV operating parameters were: step size 5 mV, pulse size 100 mV, frequency 2 Hz, and

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scan rate 10 mV s^{-1}, accumulation time 200 ms at 0.0 V. Standard stock solution of anisomycin at the concentration of 0.37 mM was prepared in methanol and stored at 4 °C. Aliquots of the stock solution were added to electrolyte to obtain concentrations in the range 0.037–14.76 nM. The total concentration of methanol did not exceed 4%. The concentration range of anisomycin used in the electrochemical experiment was selected according to reported concentrations of antibiotics in biological fluids [24].

2.3. Preparation of samples for HPLC–MS/MS analysis

The sample (5 ml) obtained in the electrochemical experiment was transferred onto clean-up cartridge (Oasis HLB, Waters, Milford, USA) preconditioned with methanol and deionized water. After drying by vacuum suction for 10 min, the analyte was eluted with 5 ml of methanol, evaporated, and reconstituted to the volume of 1 ml with methanol. The sample was filtered through 0.45 μm polyvinylidene difluoride filter (Roth, Karlsruhe, Germany) and analyzed by HPLC–MS/MS.

The urine was collected from ten healthy volunteers. The sample (1 ml) obtained in the electrochemical experiment with spiked urine was prepared as follows: 10 ml of 5% solution of TCA was added to the sample, mixture was hand-shaken for 2 min and centrifuged for 10 min at 5000 rpm. Supernatant was transferred onto clean-up cartridge (Oasis HLB) preconditioned with methanol and 5% TCA. Cartridge was then rinsed with 5% TCA, dried and eluted with 10 ml of methanol. Obtained extract was prepared for HPLC–MS/MS analysis.

2.4. HPLC–MS/MS analysis

HPLC was performed using Surveyor LC system (Thermo Fisher Scientific, Waltham, MA, USA) with reverse-phase Zorbax Eclipse® XDB-C18 column, 75 mm × 4.6 mm and 3.5 μm (Agilent Technologies, Santa Clara, USA). The mobile phase consisted of deionized water (69%), methanol (30%), and 10% acetic acid (1%). After 5 min, column was rinsed with methanol and the initial conditions were re-established and held for 10 min. The flow rate of the mobile phase was 0.4 ml min^{-1}. Injection volume was 10 μl.

HPLC system was coupled to LTQ XL (Thermo Fisher Scientific) linear ion trap mass spectrometer equipped with an electrospray ionization source, operating in the positive ion mode. Fragmentation reaction of the protonated molecular ion of anisomycin (m/z 266) to the most intensive fragment ion (m/z 206) was selected for quantification in the selected reaction monitoring (SRM) mode. The optimized MS/MS conditions were: source voltage (5.0 kV), capillary temperature (300 °C), and sheath gas (47 au, in 0–100 range defined by the LTQ XL system).

3. Results and discussion

3.1. Cyclic voltammetry

The CVs (subsequent scans, 1–20) of anisomycin on gold electrode in 0.05 M $NaHCO_3$ alongside the voltammetric response of Au electrode in blank solution (dot line) are presented in Fig. 1a. In the presence of anisomycin, the CV in the first cycle was changed so that an apparent

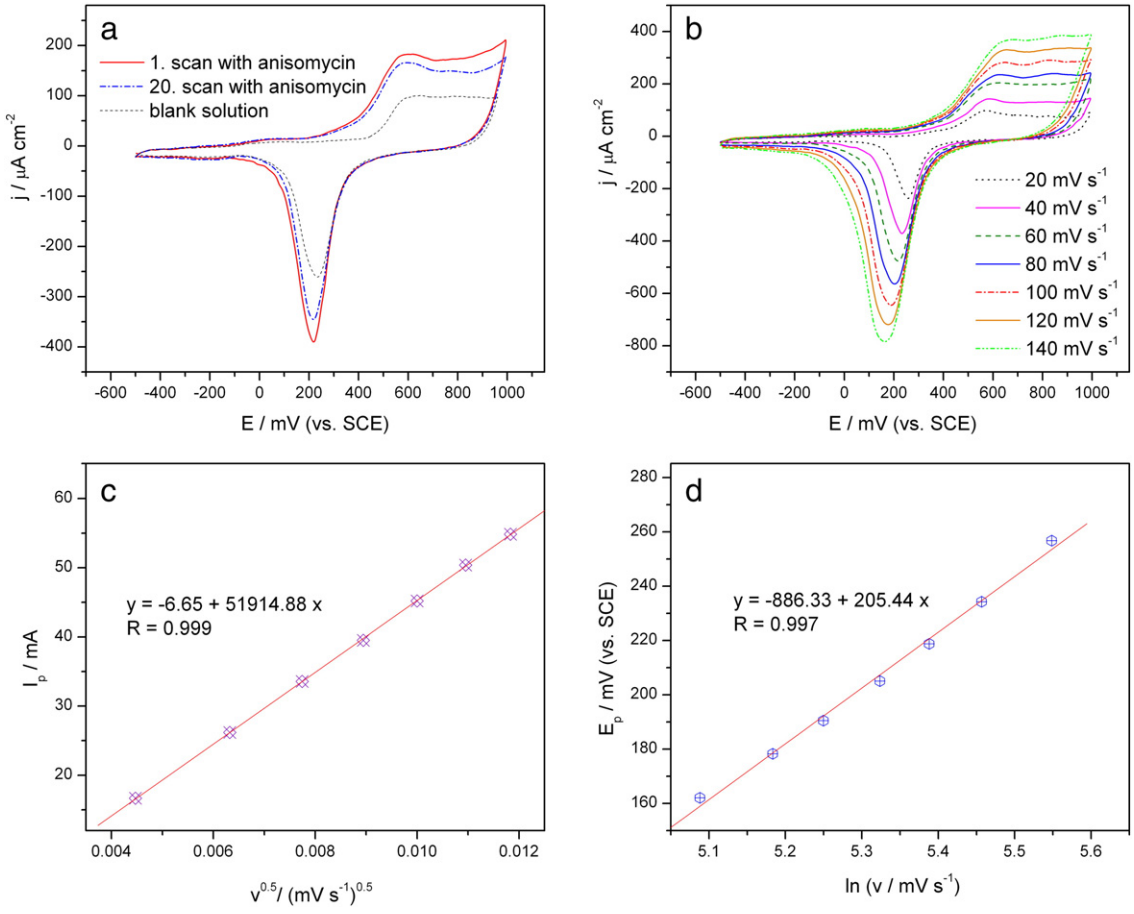

Fig. 1. Cyclic voltammogram of Au electrode using 0.05 M $NaHCO_3$ (—) and with 0.037 nM anisomycin, (a) 1. and 20. scan, scan rate: 50 mV s^{-1}, (b) for scan rates: 20, 40, 60, 80, 100, 120, and 140 mV s^{-1}, (c) plot of peak current vs. $v^{1/2}$, (d) plot of peak potential shift vs. ln of scan rates.

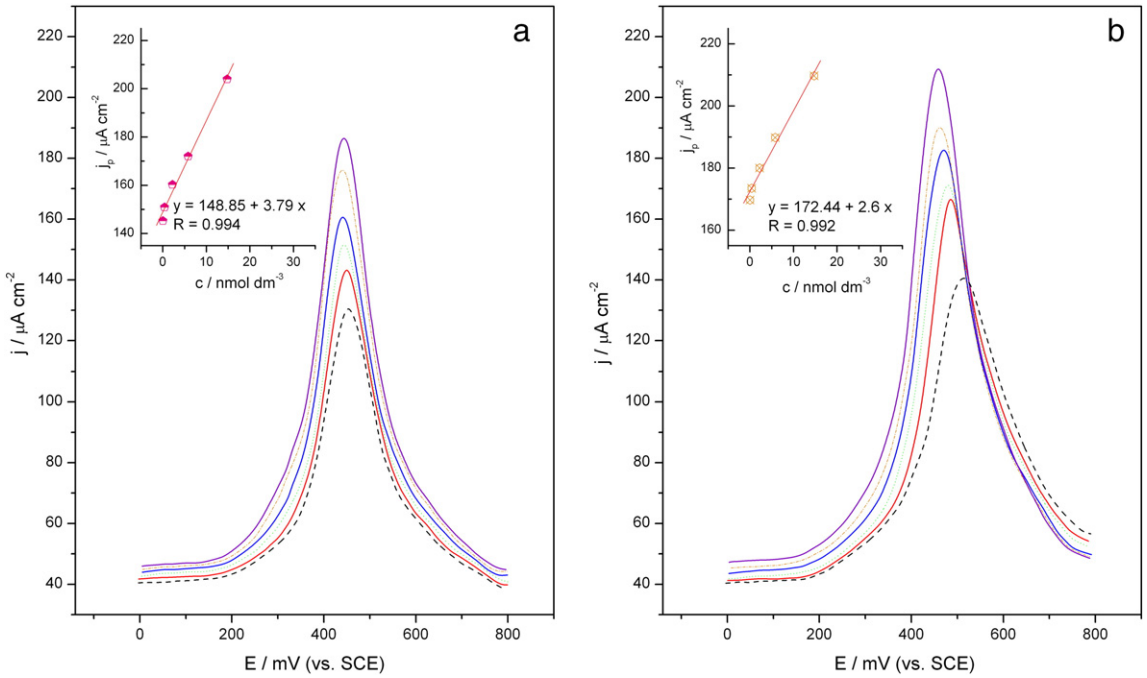

Fig. 2. Square wave voltammograms on Au electrode (—) using 0.05 M $NaHCO_3$ and with anisomycin (0.037, 0.4, 2.23, 5.84, 14.77 nM) in the absence of biological sample (a) and in the presence of biological sample (b). Step size 5 mV, pulse size 100 mV, frequency 2 Hz and scan rate 10 mV s^{-1}, accumulation time 200 ms at 0.0 V. Inset: linear dependency of anodic peak currents vs. concentration of anisomycin.

current increase occurs in the whole region of the oxide formation and reduction. The changes induced by continuous cycling are expected to be correlated with the surface oxide formation [25]. During the cycling between 1st and 20th cycles, the voltammograms show a slight decrease of anodic currents in the area of the oxide formation.

Fig. 1b demonstrates the CVs of anisomycin containing solution at different scan rates (v). The current density increases with the increased scan rate. In Fig. 1c the relationship between peak current and $v^{1/2}$ is displayed showing linearity, indicating that the anisomycin oxidation is diffusion controlled process. Furthermore, the peak potential increases with increasing scan rate, and a straight line relationship is observed between peak potentials and ln of scan rates (Fig. 1d), suggesting that the anisomycin oxidation is an irreversible electrode process [26].

According to the results presented in Fig. 1b, the kinetic parameters for the anisomycin oxidation are estimated from Laviron's theory [27]. The value of αn is calculated from the slope of E_p vs. log v. The number of electrons transferred in the electrooxidation of anisomycin was calculated to be 1.1 (approximately equal to 1) assuming that the first

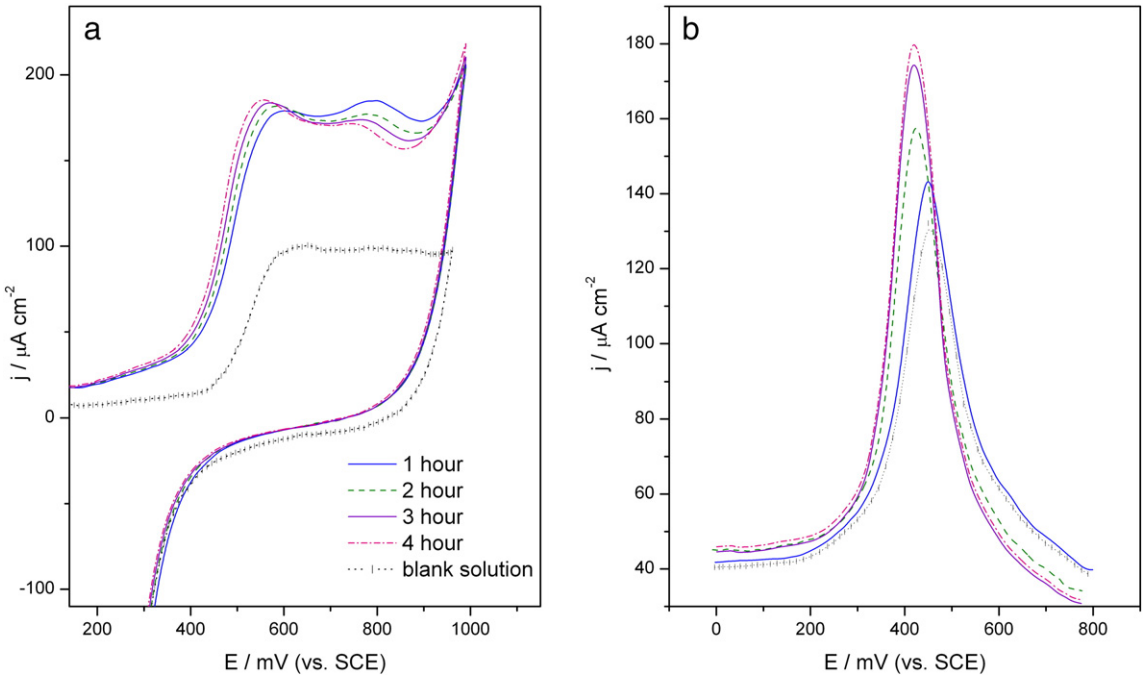

Fig. 3. Cyclic voltammograms (a) and square wave voltammograms (b) of Au electrode in 0.05 M $NaHCO_3$ (—) and with 0.037 nM anisomycin, after 1, 2, 3, and 4 h of cycling, scan rate 50 mV s^{-1}. Square wave parameters: step size 5 mV, pulse size 100 mV, frequency 2 Hz and scan rate 10 mV s^{-1}, accumulation time 200 ms at 0.0 V.

electron transfer is rate-determining so the transfer coefficient is equal to the symmetry factor which is 0.5. The value of k^o (heterogeneous electron-transfer rate constant) is determined from the intercept of the previous plot if the value of E^o is known. The value of E^o is obtained from the intercept of E_p vs. *v* curve and the value is 267.2 mV. From this, k^o was calculated as 0.23 s^{-1}.

3.2. Square wave voltammetry

The application of the SWV for quantitative determination of anisomycin at five different concentrations in the absence and the presence of human urine is displayed in Fig. 2. Oxidation proceeds by formation of well defined peak at approximately 450 mV. The peak currents exhibit linear dependence on the anisomycin concentrations in the range 0.037–14.76 nM (insert in Fig. 2a). Investigated concentrations of anisomycin generate comparably higher oxidation currents and linear dependency in the presence of biological fluid in the solution (Fig. 2b). The calculated values of LOD and LOQ [28] were 1 and 4 nM, respectively, while in the presence of biological fluid the calculated values for LOD were 2 nM and LOQ 6 nM, indicating the high sensitivity of this method.

The HPLC–MS/MS method, used for confirmation of SWV results, exhibited good linearity in the observed concentration range in the absence (R = 0.999) and in the presence of biological sample (R = 0.978).

3.3. Long term potential cycling followed by HPLC–MS/MS analysis

CV during 4 h of anisomycin oxidation (Fig. 3a) shows the appearance of the new anodic reactions with the increasing currents compared to Fig. 1a, suggesting simultaneous oxidation of anisomycin and the products of its oxidation. SWV (Fig. 3b) confirms that effect showing the increasing currents of the anodic peak.

During the cycling, samples of electrolyte were collected and analyzed by HPLC–MS/MS. It was determined that anisomycin hydrolysis occurs, and deacetylanisomycin (m/z 224) [29,30] was identified as the hydrolysis product chromatographically separated from anisomycin. Fig. 4 shows SRM chromatograms and MS/MS spectra of anisomycin (Fig. 4a) and its hydrolysis product (Fig. 4b) in 0.05 M $NaHCO_3$. Anisomycin hydrolysis under the CV conditions (Fig. 3a) is presented in Fig. 4c. In the first two hours, the amount of anisomycin decreases while the amount of hydrolysis product increases. After that, the amounts of both anisomycin and the hydrolysis product decrease and by the end of the experiment (4 h) their presence was neglectable.

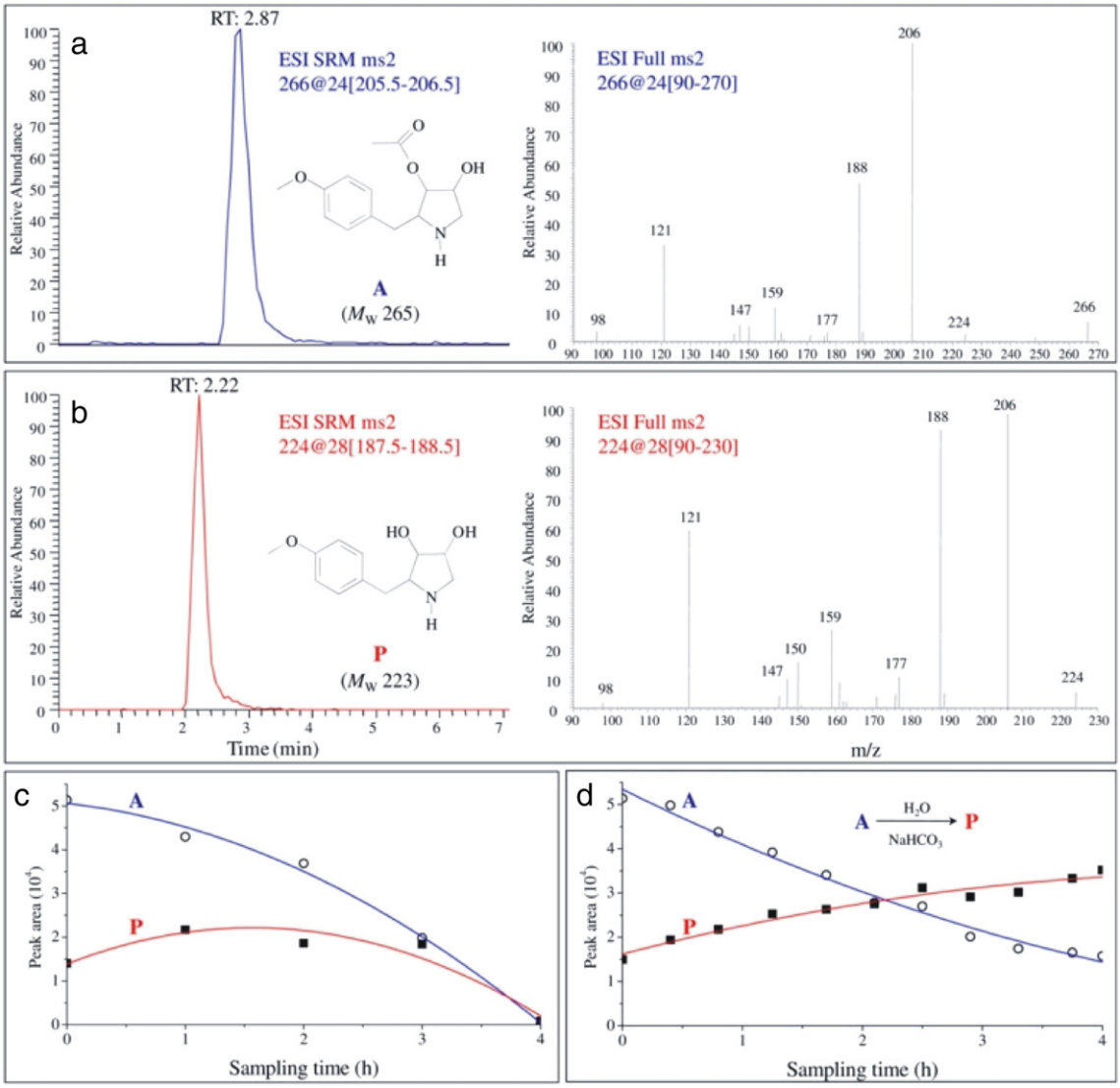

Fig. 4. SRM chromatogram and MS/MS spectrum of: (a) anisomycin, A, (b) the hydrolysis product, P. The degradation rate of A (○) and the formation of P (■) in the electrochemical experiment (c) and in 0.05 M $NaHCO_3$ (d).

This confirms that after 2 h the electrooxidation products cause the apparent increase of anodic currents in Fig. 3. It was also determined that the hydrolysis of anisomycin proceeds in 0.05 M $NaHCO_3$ without electrochemical conditions (Fig. 4d). Within the first 30 min, only 3% of anisomycin is hydrolyzed enabling the correct electroanalytical measurements for at least 30 min. Unlike the electrochemical experiment, the amount of hydrolysis product constantly increases and in 4 h only 69.5% of anisomycin is hydrolyzed.

4. Conclusions

Anisomycin, an immunosuppressant in low doses (<0.1 μM) with possible application in treatment of autoimmune diseases and in inhibiting the transplantation rejection, was electrochemically investigated for the first time. It was determined by CV at gold electrode in 0.05 M $NaHCO_3$ using its electrochemical activity. The CV experiments at different sweep rates show that process is irreversible and diffusion controlled. Based on SWV, the calculated values of LOD and LOQ were 1 and 4 nM (in the absence of biological fluid), and 2 and 6 nM (in the presence of biological fluid) indicating the high sensitivity of the proposed electroanalytical method. HPLC–MS/MS was used for quantification of anisomycin and structural identification of its hydrolysis product (deacetylanisomycin). In the first 2 h of long term cycling, the amount of anisomycin decreases while the amount of hydrolysis product increases and by the end of the experiment (4 h) their presence was neglectable.

Conflict of interest

There is no conflict of interest.

Acknowledgments

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