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Voltammetric behavior of erythromycin ethylsuccinate at a renewable silver-amalgam film electrode and its determination in urine and in a pharmaceutical preparation

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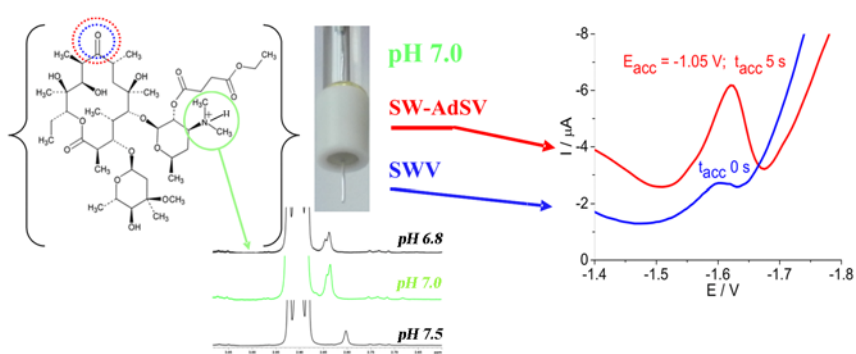
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Graphical abstract

Highlights

- Voltammetric characterization of erythromycin ethylsuccinate (EES) on Hg(Ag)FE
- Trace level determination of EES by electroreduction based SWV and SW-AdSV methods
- Protonation of the tertiary amino group supports the adsorption of EES on Hg(Ag)FE
- ^{1}H NMR confirms chemical shifting of tertiary amine methyl proton signals with pH
- Comparative HPLC-DAD measurements were performed for the validation of the methods

Abstract

Erythromycin, a macrolide antibiotic, has similar antimicrobial spectrum to penicillin and it is widely used, especially in the treatment of patients who are allergic to penicillin.

In this work, the application of a renewable silver-amalgam film electrode (Hg(Ag)FE) for the characterization and determination of erythromycin ethylsuccinate (EES), a widely used esterified form of this antibiotic, by means of cyclic voltammetry (CV) and square wave voltammetry (SWV) is presented. In the aqueous Britton-Robinson buffer (pH 5.0-9.0) that served as the supporting electrolyte, one reduction peak of EES was observed in the investigated potential range between -0.75 V and -1.80 V vs SCE, with peak potential maxima ranging from -1.59 V to -1.70 V, which strongly depended on the applied pH, as did the peak shape. For the analytical purposes the pH of 7.0 was selected, since in this electrolyte the EES peak was well-shaped and separated from the background current of the supporting electrolyte in both cases; in the direct cathodic SWV and in the case of square wave adsorptive stripping voltammetry (SW-AdSV). It was established, by the E_P-pH correlation, that protons strongly influenced the electrochemical reduction of EES. The CVs recorded between 0.025 - 0.50 V s^{-1} at pH 7.0 confirmed that the electrode reaction is adsorption-controlled. Based on the series of ^{1}H NMR measurements it is proved that the tertiary amino group of EES is mainly in its protonated form at pH 7.0 which may lead, at appropriate accumulation potential and time, to the favored adsorption of the target ionic form of the analyte improving on such way the sensitivity of the SW-AdSV method.

The optimized procedures resulted in stable SWV responses with good linear correlation in the EES concentration range from 4.53 to 29.8 μg mL^{-1} (LOD = 1.36 μg mL^{-1}), and from 0.69 μg mL^{-1} to 2.44 μg mL^{-1} (LOD 0.21 μg mL^{-1}) in the case of optimized SW-AdSV. The relative standard deviation is below 1.5%. The reliability of the elaborated procedures and thus the accuracy of the obtained results were validated by comparing them with those obtained by means of HPLC-DAD measurements. The direct cathodic SWV method was successfully applied for the determination of EES in the pharmaceutical preparation Eritromicin®, while SW-AdSV was applied in the case of the spiked urine sample. In both cases, the standard addition method was used.

Keywords: erythromycin ethylsuccinate, square wave adsorptive stripping voltammetry, renewable silver amalgam-film electrode, urine sample, pharmaceutical preparation

1. Introduction

Erythromycin, the first macrolide antibiotic discovered, has successfully been used since the early 1950s [1]. Macrolide antibiotics belong to the class of polyketide organic compounds with a lipophilic character, which feature a basic structure containing a large lactone ring and one or more deoxy sugars, most commonly cladinose or desosamine [2, 3]. Macrolides are used globally to treat a wide range of infections in human and veterinary medicine. Erythromycin is a 14-membered macrolide antibiotic produced by *Streptomyces erythreus* during fermentation and is considered the most widespread antibiotic from this group [4]. In addition, it is a precursor in the synthesis of other macrolides such as azithromycin, clarithromycin, and roxithromycin [5]. The major component of erythromycin is erythromycin A (Fig. 1A), but it also contains trace levels of erythromycin B, C and D. There are certain differences between the structure of erythromycin A and the other types; erythromycin B (12-deoxyerythromycin A) does not contain a hydroxyl group in position 12, erythromycin C lacks a methyl group in position 3", and erythromycin D (3"-O-demethylerythromycin A) contains neither a hydroxyl group in position 12 nor a methyl group in position 3". This antibiotic has a similar antimicrobial spectrum to penicillin and exhibits high activity against nearly all Gram-positive and a few Gram-negative bacteria [2, 5, 6]. It is effective for the treatment of infections induced by some intracellular pathogens, such as species of *Legionella*, *Mycoplasma* and *Chlamydia*, and is often an integral component in the treatment of upper respiratory tract as well as skin and soft tissue infections caused by different microorganisms. Erythromycin is commonly used in the treatment of patients allergic to penicillin because of its similar activity [1]. In addition, many farmers have applied erythromycin for the protection of animals and farm crops from bacterial diseases [6].

In acidic medium – which is presented in the stomach – erythromycin is unstable, forming degradation products which exhibit lower antimicrobial activity [2, 7]. Erythromycin A is relatively stable in the pH range between 4 and 10 (with degradation of less than 5% in the incubation period of 48 hours), while at pH $\leq$ 3.0 degradation is rapid with pseudo-first order rate constants of 2.36×10^{-1} and 1.30×10^{-2} min^{-1} for pH 2.0 and pH 3.0, respectively [8]. In order to prevent the degradation of erythromycin in acidic media, derivatization (esterification or addition of salts) is necessary. Two esterified forms of erythromycin – erythromycin ethylsuccinate (EES, Fig. 1B) and erythromycin estolate – are generally used. In the case of oral administration, stearate salt or esterified pro-drug forms of erythromycin are

also used, while for intravenous application erythromycin gluceptate and erythromycin lactobionate are available [9].

The incorrect use of antibiotics may leave residues in food products, thus causing undesirable effects on consumer health, e.g. allergic reactions and occurrence of resistance to certain antibiotics used in human medicine. Consequently, it is necessary to develop sensitive and reliable analytical methods for the monitoring of the concentration of antibiotics such as erythromycin in raw materials, biological samples and pharmaceutical dosage forms [10].

So far, the most frequently applied technique for the determination of erythromycin in different types of real samples (e.g. animal products, wastewater, river water, etc.) has been high performance liquid chromatography (HPLC) with tandem mass spectrometric detector [11-19]. Aside from the mentioned highly sophisticated detector, diode-array (DAD) [20, 21], chemiluminescence [22], fluorescence [23] and electrochemical [3] detectors have also been used. Additionally, spectrophotometric and conductometric methods for the estimation of erythromycin thiocyanate in both pure and pharmaceutical dosage forms were developed [24].

According to Europena Pharmacopeia 8 [25], the method of the determination of erythromycin and EES as well is based on liquid chromatography with UV detection at 215 nm. To outline this method briefly, the column is filled with a styrene-divinylbenzene copolymer, the mobile phase consists of a mixture of 450.0 mL phosphate buffer, pH 8.0 for EES and 9.0 for erythromycin, 165.0 mL 2-methyl-2-propanol, 30.0 mL acetonitrile and 355.0 mL of water, the injector volume is 100.0 µL for erythromycin/200.0 µL for EES, and the recommended column temperature is 70°C.

As a possible alternative to the above mentioned procedures for the detection/determination of erythromycin, voltammetric techniques can also be used. Different types of voltammetric techniques are especially suitable for the monitoring of a wide range of electrochemically active organic compounds of biological importance, due to their fast rate of determination, sensitivity, low cost and simply sample pretreatment procedures [26, 27]. Based on the available literature data, the basic erythromycin compound showed oxidation and reduction signal(s) as well, and therefore different types of working electrodes have been applied till now for its determination: mercury electrodes in the case of reduction [28, 29, 30]; and carbon based materials as screen-printed graphite electrode [10], bare pretreated glassy carbon electrode [31], glassy carbon electrode modified with acetylene black nanoparticles [32] and gold electrode [33] all in the case of its oxidation.

As for details concerning the reduction of erythromycin, a reduction peak at -1.43 V was observed using square wave adsorptive stripping voltammetry (SW-AdSV) and the

slowly dropping mercury electrode, in 0.1 mol L^{-1} ammonium acetate, pH 8.0, as the supporting electrolyte [28]. Adsorptive stripping voltammetry with linear scan mode (LS-AdSV) is also applicable for the determination of erythromycin at a hanging mercury drop electrode, while one reduction peak was obtained at -1.20 V in 0.025 mol L^{-1} borate buffer, pH 11.6, with an LOQ of 2 x 10^{-7} mol L^{-1} [30]. It has recently been reported that, when using linear scanning polarographic method, the EES showed a remarkable signal at -1.64 V on the hanging mercury drop electrode in a phosphate buffer supporting electrolyte with a pH of 7.46 [34]. The developed procedure has the following analytical parameters: LOD 7.5 µg mL^{-1}, linear range of measurements: 10-800 µg mL^{-1}, and a relative standard deviation (RSD) of 0.53%, The elaborated method found application in the determination of EES in Lijunsha tablets.

It is well-known that mercury is one of the best electrode materials, but because of its toxicity there is a tendency to substitute mercury with other non-toxic or less toxic electrode materials. One of the promising alternatives to the mercury electrodes is the group of amalgam based electrodes [35-57]. The main advantages of these electrodes are simple preparation and regeneration [26], the ability of this material to form amalgams with many metals/target ions, and the wide potential window in the negative potential range. A relatively new type of amalgam electrode is the renewable silver-amalgam film electrode (Hg(Ag)FE) with characteristics similar to the HMDE. The construction of the electrode provides the means to renew the amalgam film before each measurement and allows this process to be automated. Moreover, the renewal of the amalgam-film-based sensor surface usually provides good reproducibility and repeatability of the analytical signal [40]. It has successfully been used in the determination of trace levels of some metals, such as zinc [35, 43], copper [35], vanadium (V) [37], manganese (II) [38], molybdenum (VI) [39], uranium (VI) [40], chromium (VI) [41, 42], cobalt, nickel [45], lead, cadmium [43], gallium [36] and palladium (II) [44]. In addition, this electrode is suitable for the determination of different organic compounds [46-52, 56, 57] with characteristic electroactive groups in their structures, such as nitro compounds [46-48], guanidine [49, 50], quinone [57], some vitamins [56], pharmaceuticals like moroxydine [49] and doxorubicin [57], pesticides, e.g. neonicotinoid insecticides [46-48] and the fungicide blasticidin S [50] via direct voltammetric or adsorptive stripping voltammetric procedures.

The aim of this work was to characterize EES, a real dosage form of erythromycin, and to optimize the SWV and SW-AdSV analytical methods for its determination at the Hg(Ag)FE. The applicability of the developed direct cathodic SWV and SW-AdSV methods

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were tested for determination of EES in Eritromicin® tablets and in a spiked urine sample. The obtained voltammetric results were compared with the corresponding HPLC-DAD measurements. Additionally, ^{1}H NMR measurements were performed to investigate the possible protonation of the tertiary amino group of the EES at different pH values, via the chemical shifts changes of the methyl protons of the mentioned amino group, which could have significant influence concerning the SW-AdSV measurements.

2. Experimental

2.1. Reagents and solutions

All chemicals used were of analytical reagent grade. The stock solution of EES (the standard contained of 79.82% of erythromycin in form of EES) of 117.6 µg mL^{-1} was prepared by dissolving appropriate amounts of EES, kindly provided by Hemofarm a.d., Pharmaceutical - Chemical Industry (Vršac, Serbia) in the mixture of double distilled water and methanol (80% : 20%, V/V). Solution of pharmaceutical product of EES, Eritromicin® tablets (based on the declaration one tablet contained 250 mg of erythromycin in the form of EES, Hemofarm, Pharmaceutical-Chemical Industry a.d., Vršac), concentration of 294.1 µg mL^{-1}, was prepared dissolving the appropriate amount of tablet form in the mixture of double distilled water and methanol (65%: 35%, V/V).

For voltammetric measurements Britton-Robinson buffer solutions were prepared from a stock solution containing 0.04 mol L^{-1} phosphoric acid (Merck, Darmstadt, Germany), boric acid (Merck) and acetic acid (Merck), respectively, by adding 0.2 mol L^{-1} sodium hydroxide (Merck) to obtain the required pH values, covering the pH range of 4.0-9.0.

For the HPLC-DAD measurements phosphate buffer pH 8.0 was prepared from 0.02 mol L^{-1} dipotassium hydrogen phosphate (Merck) and cc. phosphoric acid (Zorka Pharma, Šabac).

The samples for the ^{1}H NMR measurements were prepared in the presence of the Britton-Robinson buffers (pH: 6.4; 6.8; 7.0; 7.5 and 8.0) with an appropriate volume of D$_2$O (Merck) 50% : 50%, V/V. In such solution 2 volume % of methanol (Merck) was added and the NMR reference standard DSS (4,4-Dimethyl-4-silapentane-1-sulfonic acid) too.

2.2. Apparatus

Voltammetric experiments were performed on an AUTOLAB PGSTAT12 electrochemical analyzer operated via GPES 4.9 software (Metrohm). The cell/vessel stand included a three-electrode system with a renewable Hg(Ag)FE (MTM Anko Instruments, Cracow, Poland [41]) of a 12-mm^2 surface area as working, a saturated calomel electrode as reference, and a platinum auxiliary electrode. All potentials are quoted vs. SCE reference electrode.

Comparative HPLC measurements were performed on an Agilent 1290 Infinity liquid chromatograph (Agilent Technologies Inc.), Zorbax Eclipse Plus C18 (2.1 mm x 50 mm, 1.8 μm) column (Agilent) and DA-detector (Agilent 1290).

The ^{1}H NMR spectra were recorded on a Bruker AVANCE III HD 400 MHz spectrometer (Bruker), equipped with Prodigy cold probe.

The pH measurements were made by using a combined glass electrode (Jenway), on a previously calibrated pH-meter (Radiometer).

2.3. Procedures

2.3.1. Voltammetry

2.3.1.1. Characterization and determination of erythromycin ethylsuccinate in model solutions

The SWV and cyclic voltammetric (CV) studies of the behavior of EES were carried out in the Britton-Robinson buffer supporting electrolytes pH 4.0-9.0. In case of the model system, the appropriate volume of EES stock solution was added by micropipette into the voltammetric vessel with double distilled water (5.0 mL) and corresponding Britton-Robinson buffer solution (5.0 mL). The surface of the Hg(Ag)FE required pretreatment before use [46, 48, 57]. It was cleaned with 2% HNO_3 for about 5 min and then covered again with amalgam by dipping it into the attached amalgam pool before each set of measurements. Furthermore, before starting measurements, the Hg(Ag)FE was electrochemically activated in the chosen supporting electrolyte by cycling its potential in the range from -0.20 to -1.60 V [46, 57] during 20 cycles. Between consecutive measurements, the sensor surface was renewed by covering it with amalgam from the pool. In the case of cyclic voltammetric experiments the scan rate was between 0.025 and 0.50 V s^{-1} in potential span from -0.75 V to -1.80 V with negative ongoing polarization, and the supporting electrolyte was Britton-Robinson buffer pH

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7.0. As for SW-AdSV, the E_{acc} values between -0.50 V and -1.50 V were investigated and for another key parameter the t_{acc} values from 0 s to 60 s were tested. In the case of the both voltammetric methods the LODs and LOQs were evaluated as the EES signal-to-baseline noise ratio of three and ten, respectively.

2.3.1.2. Determination of erythromycin ethylsuccinate in spiked urine sample

A fresh urine sample collected daily from healthy young female volunteer was analyzed without any conservation or other sample preparation steps. Determination of EES in spiked urine sample was performed by standard addition method. 100.0 µL of urine sample was added into the voltammetric vessel which contained 5.0 mL of Britton-Robinson buffer pH 7.0 and 5.0 mL of double distilled water. Spiked volume of EES with final concentration in the voltammetric vessel was 1.37 µg mL^{-1}. After recording the voltammogram for such prepared model system, three consecutive standard additions were performed with following final concentrations of EES: 0.195; 0.389 and 0.583 µg mL^{-1}. The measurement parameters in SW-AdSV were as follows: adsorption potential -1.05 V, adsorption time 5 s, quite time 5 s pulse amplitude 20 mV, step potential 5 mV, and step frequency 50 Hz.

2.3.1.3. Determination of erythromycin ethylsuccinate in pharmaceutical preparation

Standard addition method was also used for determination of EES content in Eritromicin $^{®}$ tablets. Appropriate volume of the solution of tablet form was injected into the voltammetric vessel contained 5.0 mL of Britton-Robinson buffer pH 7.0 and 5.0 mL of double distilled water. In the next steps four standard additions of EES were performed with following final concentrations in the voltammetric vessel: 2.85; 5.43; 7.81 and 9.99 µg mL^{-1}. The measurement parameters in SWV were as follows: pulse amplitude 20 mV, step potential 5 mV, and step frequency 50 Hz.

2.3.2. Liquid Chromatography

The conditions of reversed phase HPLC-DAD measurements were: mobile phase-mixture of phosphate buffer pH 8.0 and acetonitrile (45% : 55%, V/V), flow rate 0.2 mL min^{-1}, injected volume of sample 20 µL, column temperature 25 °C and working wavelength of the detector 205 nm with reference wavelength at 500 nm. Samples were filtered before measurements through the syringe micro filter Millipore 0.22 µm (Millex). The LOD and

LOQ values were evaluated as the EES signal-to-baseline noise ratio of three and ten, respectively.

2.3.3. ^{1}H NMR measurements

The ^{1}H NMR measurements were performed in water suppressed working mode. The samples were prepared from the Britton-Robinson buffers (pH: 6.4; 6.8; 7.0; 7.5 and 8.0) with appropriate volume of D$_2$O and methanol. In any case the concentration of EES was 24 mg mL^{-1}. The EES solutions for analysis were prepared from commercial form of Eritromicin$^®$ tablets and were filtered with hydrophilic syringe filters (Millex, 0.22 μm). DSS was used as the internal standard for calibration. The ^{1}H NMR measurement parameters were as follows: the standard Bruker pulse program (zgpr) was used for the water suppression, spectral window = 4800 Hz, 90° pulse length 11.75 μs, 128 scans, sample temperature 298 K.

3. Results and discussion

The experiments encompassed the SWV and CV characterization of EES in the Britton-Robinson supporting electrolyte (pH 4.0-9.0) at the Hg(Ag)FE, the optimization of the analytical procedures for its determination by means of direct cathodic SWV and by SW-AdSV in model solutions, and finally the utilization of the developed methods for the determination of the target analyte in a tablet form (Eritromicin$^®$) and in a spiked urine sample.

3.1. Voltammetric characterization of EES at the Hg(Ag)FE

Since EES has remarkable signal at pH 7.46 with maxima at -1.64 V on Hg electrode [34] this macrolide derivative was investigated via basic SWV and CV methods at the widely used contemporary form of mercury-based electrodes, the Hg(Ag)FE. In the investigated fairly negative potential range from -0.75 V to -1.80 V, one reduction peak was obtained in the pH range of 5.0-9.0, while at around 4.0 no reduction peak was observed probably because of signal overlapping with hydrogen evaluation in such acidic media. Considering the fact that the investigated erythromycin was formulated in its esterified form as ethylsuccinate, the SWV characterization of succinic acid was also performed in the first step, under the same

experimental conditions as for EES, and the results for pH=7.0 were presented in Fig. 2. This Figure depicts the comparison of SW voltammograms of the EES and succinate solutions with the voltammogram of the blank containing the Britton-Robinson supporting electrolyte (pH 7.0). As can be seen the addition of 30 μg mL^{-1} of succinic acid (curve 2) to the supporting electrolyte did not change the voltammogram of the blank (curve 1). On both voltammetric curves one broad reduction peak was observed between -0.90 and -1.40 V, with the maximum at ca. -1.2 V. A similar peak was also observed earlier in the acetate buffer with a pH of 4.5 and phosphate buffer with a pH of 7.5, and this peak is the characteristic of the Hg(Ag)FE in such supporting electrolytes [47]. After the addition of EES to the Britton-Robinson supporting electrolyte (pH=7.0), a new, well-developed reduction peak was observed with E_p = -1.67 V; this peak was separated from the signal of the blank.

The pH of the supporting electrolyte strongly affects the shape and value of the peak current of the SWV reduction signal of EES. It is most likely that at pH < 5 (not showed), the peak of the target compound is completely overlapped by the current of the reduction of hydrogen ions. As it is depicted on Fig. 3 in the pH range from 5.0 to 6.0 the EES peak ($E_p \approx$ -1.7 V) is poorly distinguished as a result of strong overlapping with the signal of hydrogen evolution. Higher pH values of the supporting electrolyte – between 6.8 and 9.0 – ensure a wider potential window till -1.75 V at the Hg(Ag)FE (see Fig. 3A), which allows well-developed SWV signals of the target analyte in this fairly negative potential range (see Fig. 3B). Increasing the pH of the investigated solution beyond this value diminishes the peak current of EES; for example, in a slightly alkaline media with a pH of 7.5 the EES peak current value is only half of that obtained at a pH of 7.0. Concerning the peak shape and intensity at pH 6.8 and 7.0, the signal in the neutral media is shifted with 20 mV positively vs. the signal at 6.8, and the potential window is wider in this supporting electrolyte with around additional 20 mV. This resulted in the best-shaped reduction peak of EES at pH=7.0.

The peak potential of EES at the Hg(Ag)FE shifts to more positive potential values from -1.70 to -1.59 V with increasing pH of the supporting electrolyte (Fig. 4), which is in accordance with earlier reported phenomena of EES on Hg electrode [34]. For pH values that are close to 7, the slope of the E_p of EES vs pH plot changes, and therefore the pH dependence at the Hg(Ag)FE can be expressed by two equations: E_p = -1.735 V + 0.0066 V/pH x pH (r = 0.987) and E_p = -1.981 V + 0.043 V/pH x pH (r = 0.998) between pH 5.0 and 6.8 and 6.8 and 9.0, respectively. It can be underline that very similar results were obtained for EES on the hanging mercury drop electrode in the phosphate buffer supporting electrolyte between pH 6.83 and 9.34 [34], wherein the positive shift of the peak potential was also

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observed with the increasing of the pH. In this paper reported slope of the peak potential and pH chart was 0.04246, which is in full agreement with the slope of the analogues correlation curve between 6.8 and 9.0 in the case of Hg(Ag)FE. As the possible reason of the positive shift of the peak potential on Hg electrode with increasing pH, the double layer capacitance change was considered, because in a neutral salt it increases [34], and in the case of Hg(Ag)FE, the reason for the described phenomena may be very similar. As it can be seen from the inset in Fig. 4, the reduction signal decreases significantly when the pH of the supporting electrolyte increases in the range between 7.0 and 9.0. Additionally, it should be emphasized that because of the overlapping character of the reduction signal caused by the target analyte with hydrogen evolution in acidic media, which results in the asymmetrical reduction signal between pH 5.0 and 6.0, it is not simple to determine the peak current maximum. Based on the above it can be concluded that protons are involved in the reduction mechanism. In any case, the most reliable reduction signal of EES was obtained at pH = 7.0 – taking into consideration the symmetry of voltammetric curve and the sensitivity – which can be thus considered the optimal value. For the basic electrochemical mechanism of the reduction of the target analyte at pH 7.0 the general scheme of keto group reduction can be considered in accordance with literature data concerning the reduction of carbonyl groups [58], and considering the fact that the number of the exchanged protons and electrons in the pH range between 6.8 and 9.0, based the slope of the E_P-pH plot which is close to the expected theoretical value of 59 mV/pH [59], is the same and thus the reduction pathway can be proposed as it is presented on Fig. 5.

Cyclic voltammetric experiments were performed to investigate the possible adsorption effect of the EES molecules at Hg(Ag)FE. The CV curves were recorded in the potential span between -0.75 V and -1.80 V with scan rate from 0.025 to 0.50 V s^{-1}, while the concentration of the EES was 25.9 μg mL^{-1} in the supporting electrolyte at pH 7.0. As in the case of the SWV results, only one reduction peak was observed, and this signal is without any counterpart during the potential scan in positive direction, which confirms, that in the investigated potential range at all the investigated scan rates the EES showed irreversible reduction process. This feature can be described as an adsorption controlled process because the obtained I_P values showed linear dependence from the investigated scan rates as follows: I_P = -0.0558 μA - 0.00278 μA/(mV s^{-1}) x v while the coefficient of correlation was -0.995. The appropriate correlation was presented on the Fig. 6 together with the consideration of the results of I_P-$v^{1/2}$ correlation.

Considering all of the above, it can be proposed that the EES molecules adsorb on the Hg(Ag)FE surface. Closely to pH 7 the EES is probably mainly in its protonated form via its tertiary amino group (with pKa 7.1 [60]), and the adsorption of the target analyte can be supported by positively charged EES and by the appropriate negative polarization of the electrode. In slightly alkaline media as pH 7.5 and at higher pH values the EES molecules are mainly in their neutral form, thus they have significantly lower affinity to the negatively polarized Hg(Ag)FE surface in comparison to the positively charged form of EES. To get deeper insight into the influence of the pH of the supporting electrolyte on the adsorption measurements the water suppressed ^{1}H NMR experiments were performed to investigate the possible protonation of the tertiary amino group of the EES at applied pH values, via the chemical shifts changes of the methyl protons of the mentioned tertiary amino group. The full ^{1}H NMR spectrum of EES from its pharmaceutical preparation at pH 7.0 is illustrated at Fig. 7A, and the signals of the mentioned methyl groups at different pH values ranging from pH 6.4 to 8.0 are presented as Fig. 7B. In the case of tertiary amino group the protons from the two methyl groups have a complex and in some cases almost overlapped peak form, and have significantly different chemical shifts in the protonated/positively charged and deprotonated/uncharged form of the nitrogen atom. In the case of pH 6.4, 6.8 and 7.0 the chemical shifts for the most pronounced peak maxima are as follows: 2.84, 2.83, 2.83 ppm (Fig. 7B). Significantly different values were obtained in the case of pH 7.5 and 8.0 (2.80 and 2.76 ppm, respectively), which can be explained by the fact that under such circumstances the amine nitrogen is mainly in its deprotonated form. Because in such cases the negative inductive effect of the nitrogen is not so expressed as it is in the case of protonated and positively charged form of the EES, the target protons on the methyl groups are more shielded by the electrons which is in accordance with the upfield shifts of the methyl signals (shifted to lower ppm values).

Beside of the appropriate pH of the supporting electrolyte, which probably cause the protonation of the target molecules, as it is illustrated on Fig. 8, the adsorption/accumulation potential, E_{acc}, and adsorption/accumulation time, t_{acc}, are the parameters which significantly influences the analytical signal intensity in the case of SW-AdSV experiments. It can be assumed, that at an appropriate potential value, probably the ketonic carbonyl group from the large lactone ring could be the first electroactive centre in the proton-driven reduction process.

The adsorption behavior of the target analyte was investigated at E_{acc} values between -0.50 V and -1.50 V, and at t_{acc} values from 0 s to 60 s. It was found that when E_{acc} was

between -0.50 V and -1.00 V the signal intensity was in general unchanged, but at potentials lower than -1.00 V the reduction signal intensity was changed, especially when appropriate accumulation time was selected (see on Fig. 8A). Namely, in the case of all investigated t_{acc} depending on the applied potentials the 5 or 10 s is the appropriate measurement condition. In the cases from -1.00 V to -1.10 V the shorter t_{acc} is the favorite. Although the positively charged EES can have higher affinity to the electrode surface with more negative polarization, the obtained signals suggested that at such potentials, among other possible effects, the competitive hydrogen evaluation can be expected which caused the I_p decrease. With increasing t_{acc} the same effect can result the reduction signal decrease just in the case of the favored E_{acc}. The selected voltammetric curves, obtained at SWV (curves marked as 0 s) and SW-AdSV (curves marked as 5; 10 and 60 s) working modes at different E_{acc} are presented on Fig. 8B-D, the red curve (Fig. 8B) presents the signal obtained under optimized conditions. The presented curves served as illustration of qualitative behavior of the system, especially in the case of the direct SWV signals because under such investigated conditions the EES concentration is between the LOD and LOQ of the direct SWV method.

These findings are in agreement with the earlier reported results of electrocapillary curve experiments, and the cationic, neutral and anionic surfactants influence on the reduction signal of EES, both sets of experiments reported by Zhang and Tuo at Hg electrode [34], where it was elaborated that the EES significantly reduce the surface tension of the drop, which indicating its adsorption, and the positive surfactant influence on the reduction peak decrease suggest that the EES molecule is adsorbed in its positive form.

3.2. Development of direct cathodic SWV and SW-AdSV methods at Hg(Ag)FE for determination of erythromycin ethylsuccinate

After the selection of the appropriate pH of the supporting electrolyte as pH 7.0, which is one of the key parameters regarding to the method's sensitivity, and basic optimization of SWV parameters (not shown) the here presented direct cathodic SWV analytical method was developed. Under the optimized conditions, the SWV determination of EES was based on the linear relationship between the I_p of EES and the target analyte concentration from 4.53 to 29.8 μg mL^{-1} (Fig. 9A, the inset showed the linear range of the calibration curve). The analytical parameters of this determination of EES are summarized in Table 1. The reproducibility of the method was estimated via six successive measurements of the solution containing 10.7 μg mL^{-1} EES (Fig. 9B) and the relative standard deviation (RSD) was found

to be 0.84%. In the case of the SW-AdSV measurements based on the optimized t_{acc} and E_{acc} parameters as 5 s at -1.05 V, the analytical method was developed with the remaining operating parameters as in the case of direct cathodic SWV. The obtained voltammograms (Fig. 9C) showed that there is a linear correlation between the concentration of EES and I_p from 0.69 to 2.44 µg mL^{-1} (inset), at higher concentration the calibration curves showed saturation. The RSD of the method, based on the six times repeated SW-AdSV signals for 1.09 µg mL^{-1} of EES at pH 7.0 (Fig. 9D) is 1.2%. Thanks to the adsorptive type of measurement the LOD of the method is lowered around six times. The appropriate SWV-AdSV analytical parameters are given in the Table 1 as well.

Comparative HPLC-DAD measurements were also performed. The optimized HPLC-DAD method was applicable in a concentration range of EES that was twice as wide – from 1.18 to 58.8 µg mL^{-1} – in comparison to the direct cathodic SWV one (for details see Table 1). The RSD of the method for 10.0 µg mL^{-1} of EES is 1.1%. Even though the HPLC-DAD methods very often have significantly lower LOQ values, in this case, because of the lack of the sensitive chromospheres the selected DAD working wavelength of 205 nm, the determination of target analyte is just with four times lower LOQ as it is the case of the developed direct SWV method, and nearly two times higher as it is case for SW-AdSV. Additional analysis of the recorded UV spectra suggest the 210 and 215 nm as the next possible working wavelengths, but after the optimization of the method, these wavelengths were found as the possible choice with a little bit higher LOQ value of the method.

Although the HPLC-DAD method is applicable in wider concentration range, the developed voltammetric methods have some advantages such as suitability for the on-site analysis, requirements for very simple sample preparation procedures, low cost, fast response time and in the case of SW-AdSV, a lower LOQ.

3.3. Determination of erythromycin ethylsuccinate in selected samples

The elaborated voltammetric methods were tested for the determination of EES in the spiked urine sample (SW-AdSV) and in the tablet form Eritromicin® (SWV). In the case of both type of samples the standard addition method was selected for the determination of the concentration/amount of active compound.

3.3.1. Determination of erythromycin ethylsuccinate in spiked urine sample

The accuracy of the elaborated SW-AdSV method employing the Hg(Ag)FE was assessed by quantifying EES in a solution containing 10.0 mL of twice-diluted Britton-Robinson buffer (pH 7.0), which was spiked with 100.0 µL of human urine and a known amount of 1.37 µg mL^{-1} EES (final concentration in voltammetric vessel). The sample was analyzed using the standard additions procedure because the matrix reduces the EES signal intensity. A typical standard additions plot and the series of SW-AdSV curves obtained after standard additions are presented in Fig. 10.

The contents of EES obtained in three repeated measurements (1.33, 1.36 and 1.47 µg mL^{-1}, average EES content 1.39 µg mL^{-1}, RSD 5.3%) are very close to the spiked values, which are equal to 1.37 µg mL^{-1}. Beside the optimization of t_{acc} and E_{acc} of the SW-AdSV additional simple sample preparation steps as liquid-liquid, or solid phase extractions can lead to the further improvement of the sensitivity of the method.

3.3.2. Determination of erythromycin ethylsuccinate in pharmaceutical preparation

After appropriate preparation of the solution from the tablet form, EES was determined in the commercial pharmaceutical product Eritromicin® at pH 7.0 by optimized SWV method at Hg(Ag)FE. In the depicted case (Fig. 11) the nominal concentration (based on the declaration of the manufacturer) of EES was 14.35 µg mL^{-1}. After recording the baseline signal (curve 1) and voltammogram of the sample of pharmaceutical preparation (curve 2) four consecutive standard additions were added (curves 3-6), so that the final EES concentration was in the range of 2.85-9.99 µg mL^{-1}, which resulted in the found amount of 14.26 µg mL^{-1} of the EES target analyte. The standard addition method was applied because the EES signal decrease was recognized, in comparison to the signals from the adequate model system, which can be explained by the matrix effect.

The average content of the erythromycin in tablet form Eritromicin®, obtained by determination of EES, and later counted to erythromycin, by developed SWV method was 251.2 mg (expressed as erythromycin) which is in good agreement with the declared content (250 mg expressed as erythromycin). Comparative reversed phase HPLC-DAD measurements were also performed, and the obtained results are elaborated in the Table 2. It was found that the reproducibility of the analytical signal, expressed as RSD is lower than 1.2 %. Having in mind the basic requirement of European Pharmacopeia 8 in term of errors in the accuracy of the measurements, both the elaborated analytical methods fulfilled these criteria, and confirmed that in the analyzed tablets the declared amount of erythromycin was found.

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Having in mind the great potential of Hg(Ag)FE further exploratory work is planned in the term of widening its possible application area especially in the case of characterization and determination of selected macrolide antibiotics and other compounds of biological and/or environmental importance.

4. Conclusions

Simple and fast SWV and SW-AdSV procedures using a contemporary renewable silver amalgam film electrode (Hg(Ag)FE) were developed for the characterization and determination of erythromycin ethylsuccinate (EES) in the Britton-Robinson buffer supporting electrolytes, and the appropriate pH value for analytical method was found as 7.0. Based on the series of water suppressed ^{1}H NMR measurements it was proved, that at the selected pH value for the electroanalytical measurements, the investigated analyte EES, mainly exists in protonated form via its tertiary amino group. This cationic form is assumable the favorite moiety in the optimized SW-AdSV measurements. The linear response was obtained in two concentration ranges, lower in the case of SW-AdSV from 0.69 to 2.44 μg mL^{-1} and higher in the case of direct cathodic SWV from 4.53 to 29.8 μg mL^{-1} – and the RSD did not exceed 1.5%. It was demonstrated that the elaborated methods, using the standard addition procedure, were useful for the determination of EES in the spiked urine sample (SW-AdSV) and in the pharmaceutical preparation Eritromicin® (SWV). The results of the comparative HPLC-DAD measurements of EES concentration in model solutions and in the tablet form Eritromicin® were in good agreement with those obtained by means of the investigated voltammetric method at the Hg(Ag)FE.

Further exploratory work is planned for expansion of application area of the Hg(Ag)FE in pharmaceutical analysis.

Acknowledgements

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Figure captions

Figure 1. Structure of erythromycin A (A) and erythromycin ethylsuccinate (B).

Figure 2. SW voltammograms of: 1) Britton-Robinson supporting electrolyte, pH=7.0, 2) as 1 + (and) 30 μg mL^{-1} of succinate (added in the form of succinic acid), and 3) as 1 + 28.5 μg mL^{-1} EES.

Figure 3. SWV signals recorded on the Hg(Ag)FE of: A) Britton-Robinson supporting electrolytes from pH 5.0 to 9.0 and B) same buffer solutions in the presence of 15.6 μg mL^{-1} EES.

Figure 4. E_p-pH and I_p-pH (inset) dependences of erythromycin ethylsuccinate (c = 15.6 μg mL^{-1}) in Britton-Robinson supporting electrolyte from pH 5.0 to 9.0.

Figure 5. Possible proposed reduction pathway of erythromycin ethylsuccinate at pH 7.0.

Figure 6. Linear plot of the reduction peak current (I_p) *vs.* the potential scan rate (*v*) from 0.025 V s^{-1} to 0.50 V s^{-1} and the illustration of the obtained results in the case of I_p *vs.* $v^{1/2}$ correlation for Britton Robinson supporting electrolyte pH 7.0 with 25.9 μg mL^{-1} EES.

Figure 7. ^{1}H NMR spectra of EES in Britton-Robinson buffer with D$_2$O at different pH values: A) The full water suppressed ^{1}H NMR spectrum of EES in D$_2$O (containing MeOH and DSS) at pH 7.0; B) Section plots for N,N-dimethyl signals at pHs 6.4, 6.8, 7.0, 7.5 and 8.0.

Figure 8. Influence of the accumulation potential and accumulation time on the reduction signal of EES (c = 1.96 μg mL^{-1}) in Britton-Robinson supporting electrolyte pH 7.0: A) comparison of the I_p obtained at different t_{acc} at 0; 5;10, 30, and 60 s at selected E_{acc} (1) -1.00; 2) -1.05; 3) -1.10; 4) -1.15 and 5) -1.20 V), and B-D some the illustrative voltammetric signals obtained at selected accumulation potentials (the selected accumulation times are marked on the curves in s) at: B) -1.05 V, C) -1.15 V and D) -1.20 V.

Figure 9. Characteristics of the developed analytical methods at the Hg(Ag)FE in Britton-Robinson buffer with pH 7.0: A) SWV signals recorded in the concentration range of EES 4.53-29.8 μg mL^{-1} (inset: corresponding calibration curve), B) reproducibility of the direct SWV analytical signals for 10.7 μg mL^{-1} EES (6 repetitions), C) SW-AdSV signals in the

concentration range of EES 0.69-2.44 µg mL^{-1} (inset: corresponding calibration curve), D) the reproducibility of the SW-AdSV analytical signals (6 repetitions) for 1.09 µg mL^{-1} EES.

Figure 10. An example of EES quantitation in the spiked urine sample by SW-AdSV on Hg(Ag)FE. Bottom to top: Britton-Robinson supporting electrolyte with urine sample (dashed line); as dashed line + 1.37 µg mL^{-1} EES and three successive standard additions with final concentrations of 0.195; 0.389 and 0.583 µg mL^{-1} EES. Inset: analytical curve.

Figure 11. Determination of concentration of EES in pharmaceutical preparation Eritromicin$^{®}$ by standard addition method. SW voltammograms of baseline (1), sample of commercial formulation (2) four standard addition of EES (3-6, final concentrations in the voltammetric vessel : 2.85, 5.43, 7.81 and 9.99 µg mL^{-1}) and the appropriate analytical curve in the inset.
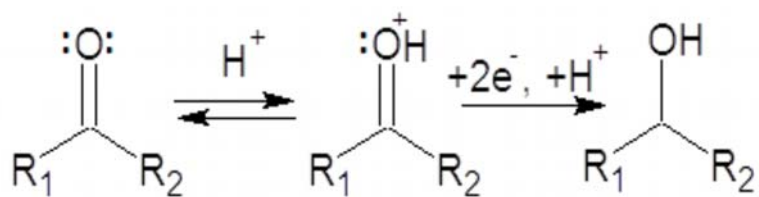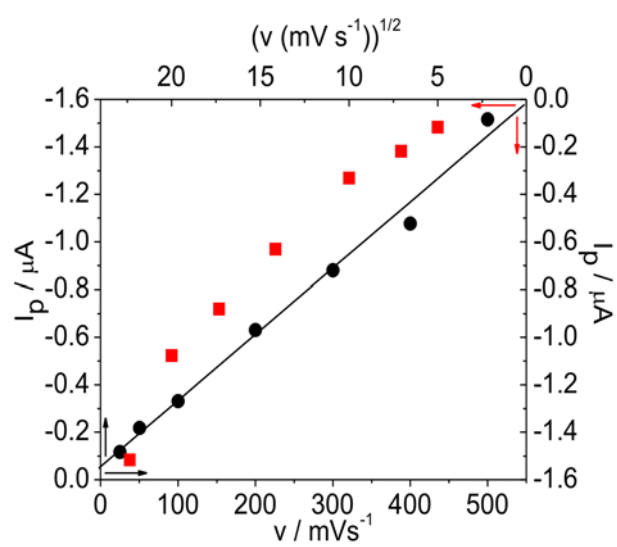
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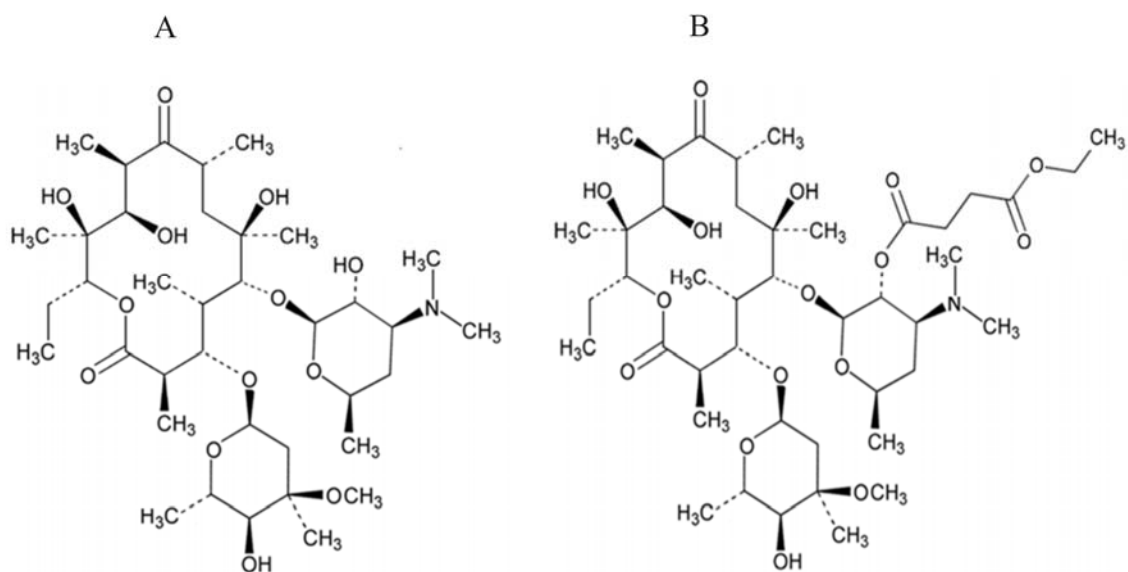
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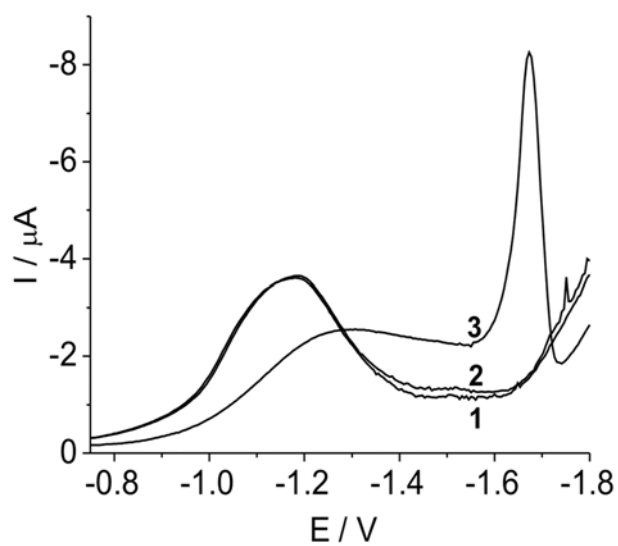
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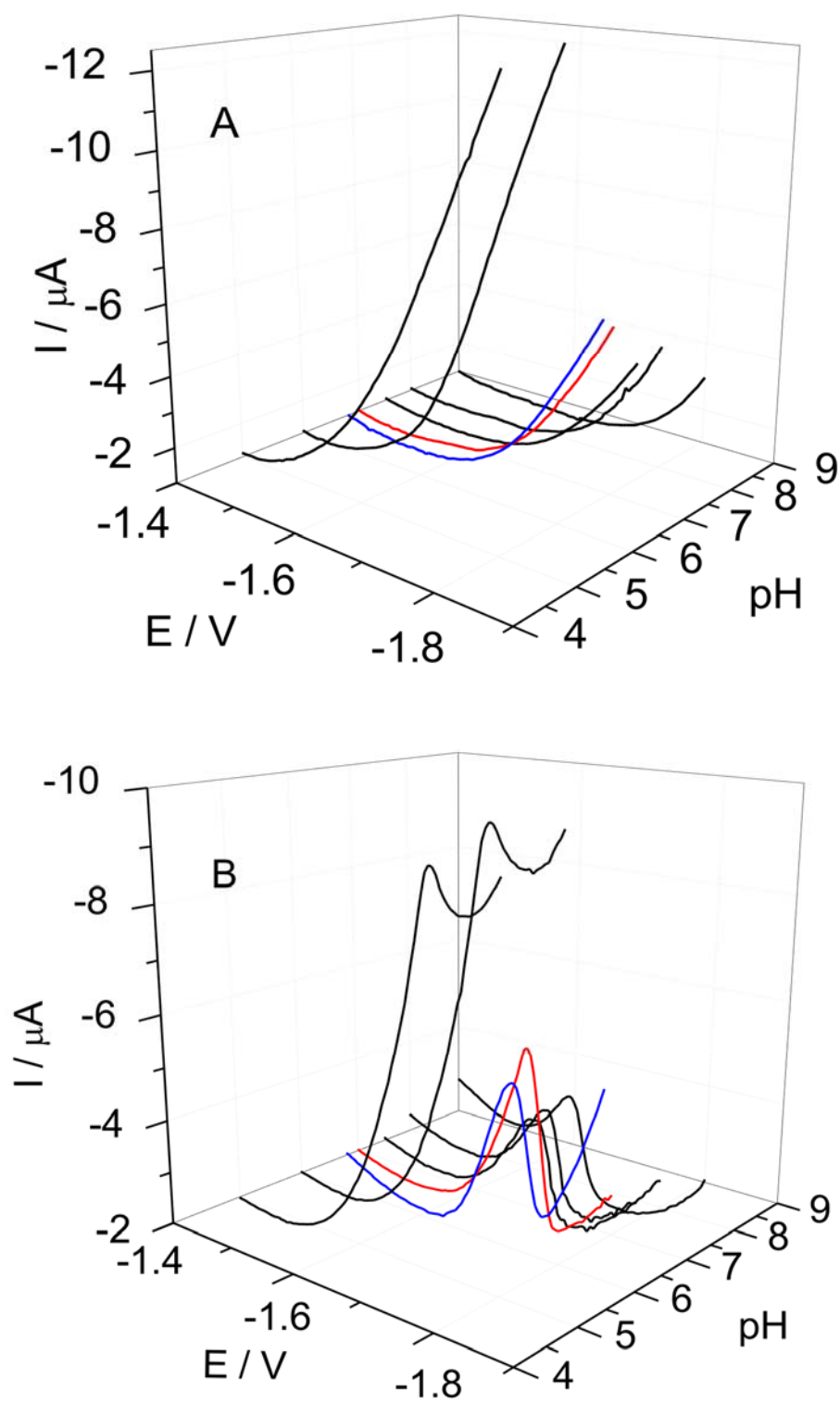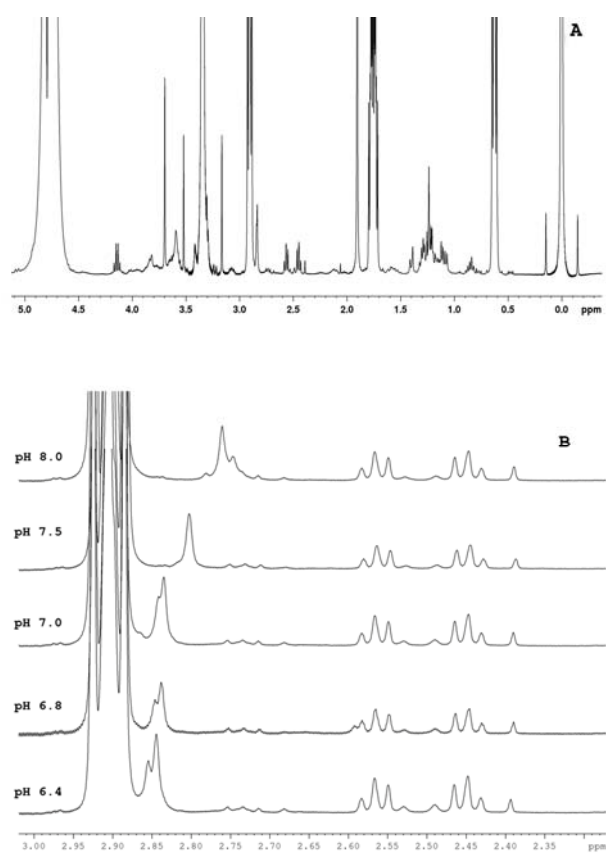
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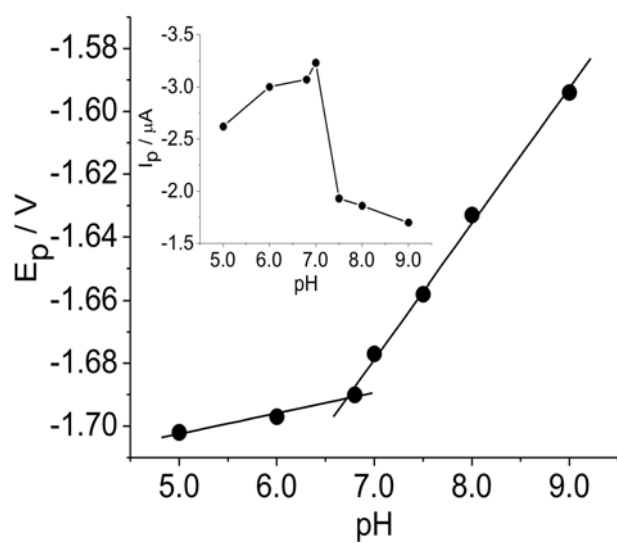
Figure 5

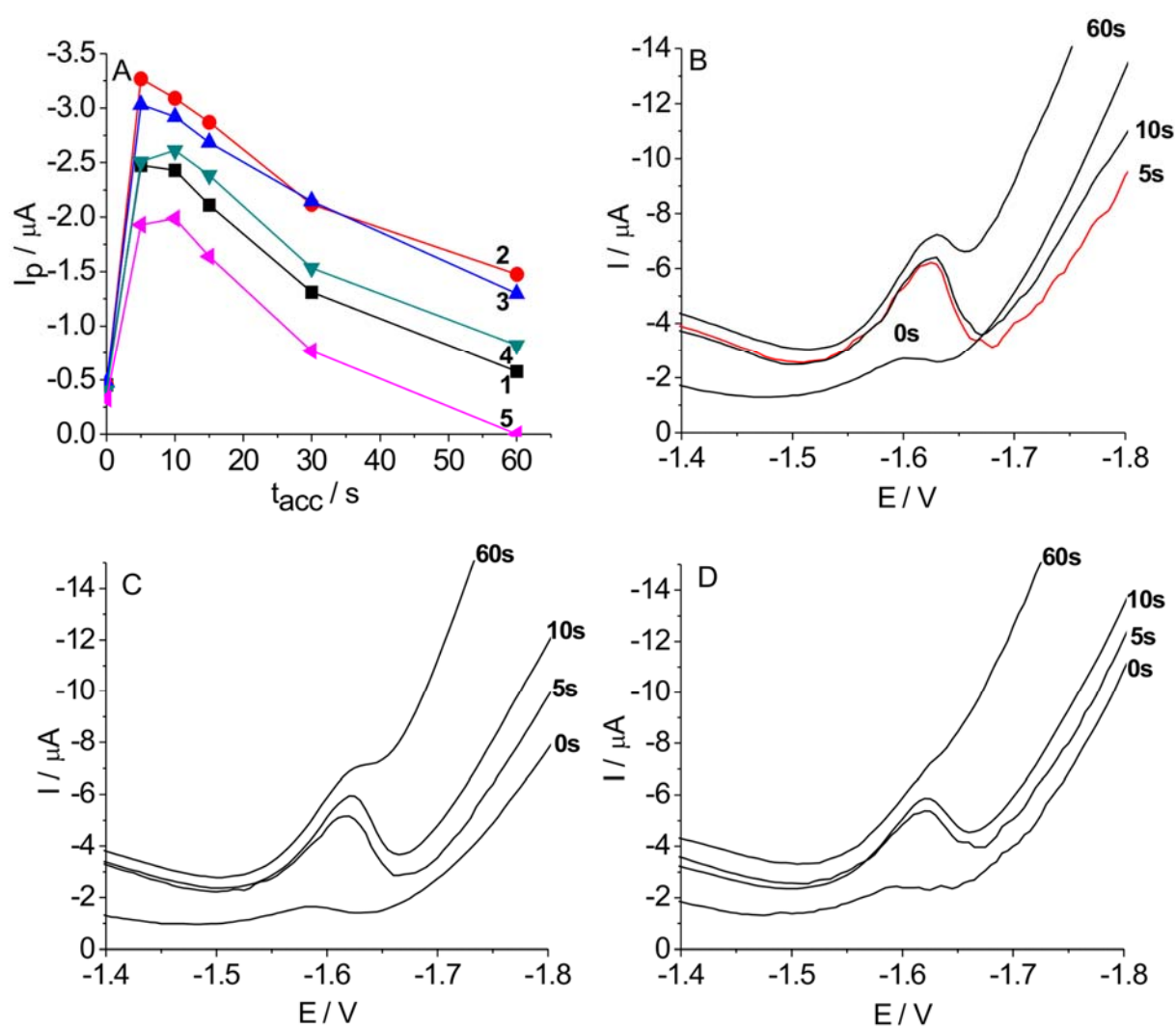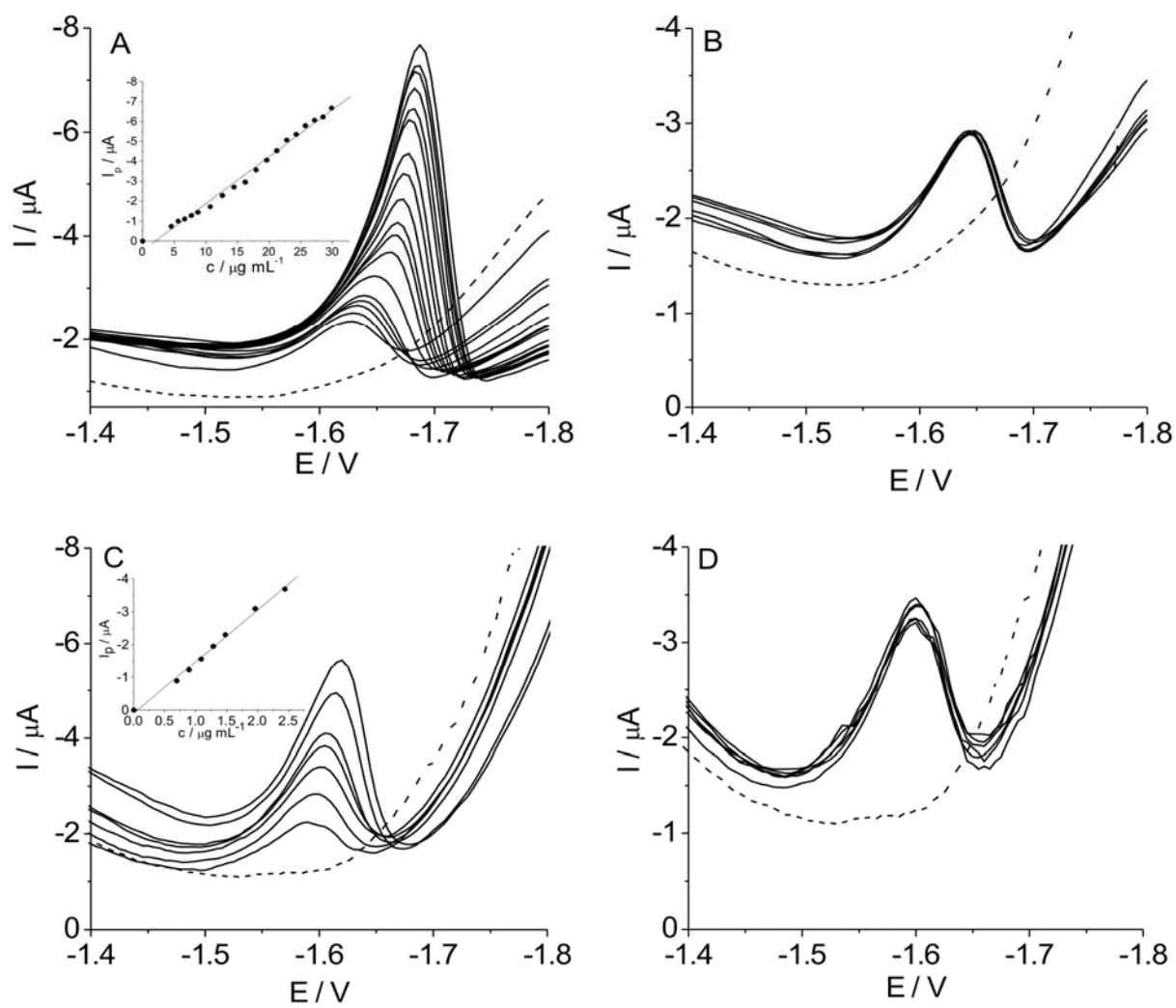Figure 6

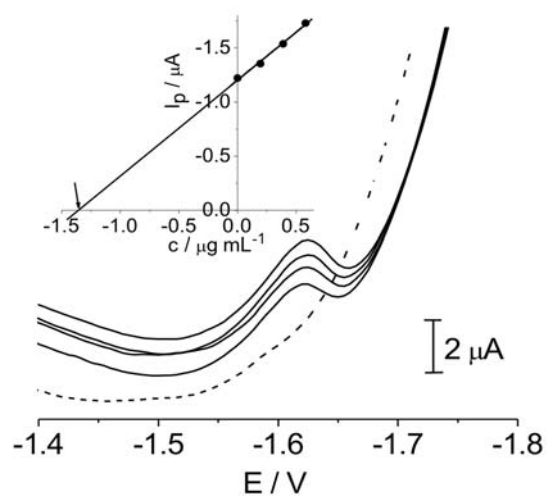Figure 7

Figure 8

Figure 9

Figure 10

Table 1. Analytical parameters for direct cathodic SWV and SW-AdSV methods obtained on Hg(Ag)FE for determination of EES together with comparative data of HPLC-DAD method, r: linear regression coefficient; LOD: limit of detection; LOQ: limit of quantitation

Parameters	Hg(Ag)FE-SWV	Hg(Ag)FE-SW-AdSV	HPLC-DAD
Linear concentration interval ($\mu g\ mL^{-1}$)	4.53-29.8	0.69-2.44	1.18-58.8
Intercept	0.473 (μA)	0.100 (μA)	-1.583 (mAUs)
Slope	-0.235 ($\mu A\ mL\ \mu g^{-1}$)	-1.577 ($\mu A\ mL\ \mu g^{-1}$)	8.163 (mAUs mL μg^{-1})
Correlation coefficient	-0.995	-0.997	0.999
LOD ($\mu g\ mL^{-1}$)	1.36	0.21	0.35
LOQ ($\mu g\ mL^{-1}$)	4.53	0.69	1.18

Table 2. Determination of erythromycin in form of EES and expressed as erythromycin in Eritromicin® tablets. The declared amount of the active compound is 250 mg expressed as erythromycin/tablet

Parameters	Found (mg/tablet) by Hg(Ag)FE-SWV	Found (mg/tablet) by HPLC-DAD
Sample number 1	248.5	252.2
Sample number 2	253.0	246.9
Sample number 3	252.1	250.5
Average value	251.2	249.9
RSD (%)	0.95	1.12