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Use of carbon paste electrode and modified by gold nanoparticles for selected macrolide antibiotics determination as standard and in pharmaceutical preparations

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This paper is dedicated to the memory of our wonderful colleague and professor, dr Valéria Guzsvány, who recently passed away.

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Abstract

In this work, carbon paste electrode (CPE) and carbon paste electrode modified with gold nanoparticles (AuNPs/CPE) were employed with rapid direct anodic square wave voltammetry (SWV) for macrolide antibiotics erythromycin ethylsuccinate (EES), azithromycin (AZI), clarithromycin (CLA) and roxithromycin (ROX) determination. The surface of working electrodes were morphologically characterized by scanning electron microscopic (SEM) measurements whereby the presence of randomly distributed AuNPs of 10 nm diameter size on CPE surface was confirmed by energy dispersive spectrometer (EDS). SWV determination of four macrolide antibiotics using CPE was performed in aqueous Britton-Robinson buffer solutions (pH 2.0 to 11.98) showing that oxidation signals strongly depend upon pH (at pH $\geq$ 6) and exhibited the most favorable characteristics at pH 8.0 in the case of EES and pH 11.98 in the case of AZI, CLA and ROX. Under optimized conditions, the SWV method using CPE enables determination of all investigated macrolide antibiotics in low μg mL^{-1} concentration ranges with relative standard deviations (RSDs) lower than 6% and achieved detection limits (LODs) as 0.18, 0.045, 1.43 and 0.30 μg mL^{-1} for EES, AZI, CLA and ROX, respectively. In the case of AZI and ROX, it was demonstrated that the use of AuNPs/CPE as working electrode could additionally improve the results obtained with the SWV method concerning exhibited sensitivity, reproducibility and linear concentration range, due to the electrocatalytic properties of synthesized AuNPs. The optimized experimental parameters with the use of SWV method and CPE or AuNPs/CPE were successfully applied for determination of ROX and AZI in their pharmaceutical preparations Runac® and Hemomycin®, respectively. 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.

Keywords: macrolide antibiotics; square wave voltammetry; carbon paste electrode; gold nanoparticles; pharmaceutical preparations

1. Introduction

The macrolide antibiotics are of great significance in medicine and in the clinical practice. Among them, erythromycin (ERY), azithromycin (AZI) and clarithromycin (CLA) have important role for treatment of respiratory tract infections [1, 2]. Thanks to many important biological properties such as antibacterial and antifungal activities, good cell penetrance, anti-inflammatory and immunomodulatory effects, macrolides are widely used in human and veterinary medicine [3-7]. The antimicrobial action of these antibiotics is based on the reversible binding to the 50S subunit of the bacterial ribosome which results in the blockage of peptide chain elongation i.e. inhibition of protein synthesis [6]. The first discovered macrolide antibiotic, ERY [1] is a precursor in a synthesis of other semisynthetic macrolide antibiotics such as AZI, CLA and roxithromycin (ROX) [8]. They contain lactone ring (12 to 16 carbon atoms) with one or more deoxy sugars linked via glycoside bonds in their structure. Instability of ERY in acidic media requires its derivatization in term of esterification or addition of salts. Therefore, the most commonly used esterified forms of ERY in clinical practice are erythromycin ethylsuccinate (EES) and erythromycin estolate [9]. Structural changes of AZI (Fig. 1B), CLA (Fig. 1C) and ROX (Fig. 1D) in comparison with structure of ERY (Fig. 1A) lead to better stability of these three macrolides in acidic media which improve their pharmacokinetic properties [10-12]. Beside their use for the treatment of respiratory tract infections [7, 13], ERY, AZI and CLA can be applied for skin and soft infections, as well. Additionally, AZI and CLA have found application for prevention and treating disseminated *M. avium* complex disease of HIV-infected patients [7].

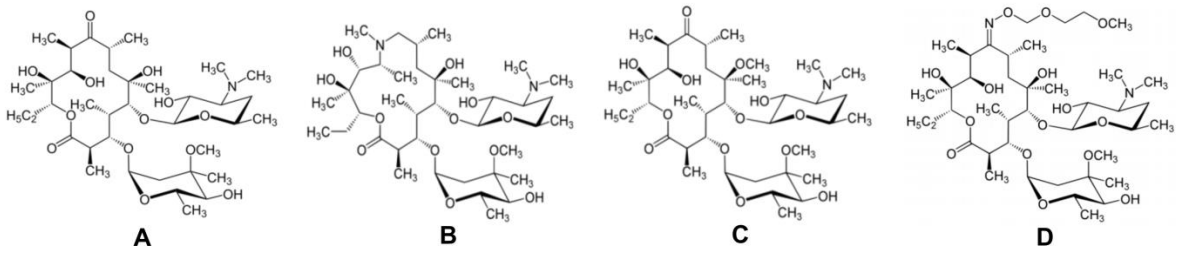

Fig. 1. Molecular structure of erythromycin (A), azithromycin (B), clarithromycin (C) and roxithromycin (D).

Macrolide antibiotics belong to the safest antibacterials because they are less toxic and the allergic reactions are very rare compared to other antibiotics [6]. The most common side effects of ERY, AZI, CLA and ROX are gastrointestinal disturbances [7, 14-16]. But, in general, incorrect use of all antibiotics can lead to resistance which is the most serious threats to human

health [16] and in the case of macrolide antibiotics it increased between 20% and 70% in certain parts of the world in the last 5 decades [17]. Because of that there is a need for development of reliable analytical methods for monitoring their concentration in different real samples.

The most commonly used technique for determination of ERY, AZI, CLA and ROX is high performance liquid chromatography (HPLC) coupled with different types of detectors [18-27]. Besides the highly sophisticated HPLC, electrochemical techniques have proven to be suitable for characterization and/or determination of mentioned macrolides [28-47]. So far, the mercury based electrodes and renewable silver-amalgam film electrode (Hg(Ag)FE) were successfully employed for determination of ERY, EES, CLA, AZI and ROX [28-34] based on their reduction while the gold and different carbon based electrodes such as glassy carbon and carbon paste electrodes (CPE) in the unmodified or modified form were used for characterization and/or determination of macrolides based on their oxidation [35-47]. In the Table 1 are given brief overview of applied methods and working electrodes together with obtained results for determination of ERY, EES, AZI, CLA and ROX.

The carbon paste is convenient electrode material due to its good electrical conductivity, low background current, applicability in wide potential range and maybe the most important advantage is its ability for simple and fast modification with numerous noble metal nanoparticles [48] which can improve the selectivity and sensitivity of the analytical methods. Besides AZI [43, 44] the CPE has proved to be applicable for determination of other different antibiotics. In unmodified form it was suitable for determination of rifampicin [49], isoniazid [49], adriamycin [50], ciprofloxacin [51], lomefloxacin [52], sparfloxacin hydrochloride [52], gatifloxacin [52] and etc. while the modified CPE was applied for determination of amoxicillin [53], cefixime [54], sulfasalazine [55], selected fluoroquinolone antibiotics [56] and others. It is well known that the noble-metal nanoparticles such as gold nanoparticles (AuNPs) have good catalytic [57] and unique electronic [58] properties and therefore are widely used for design of different electrochemical sensors, as well as for miniaturization of sensing devices to the nanoscale [59]. Moreover, the AuNPs modified working electrodes are proven to be suitable for electrochemical determination of different organic compounds such as glucose [60-62], caffeine [63], dopamine [64-66], serotonin [65], hydrazine [67], epinephrine [68, 69], paracetamol [64], 4-aminophenol [64], quercetin [70], uric [66, 71, 72] and ascorbic [71, 72] acids, etc. CPE surface modified with

gold nanoparticles (AuNPs/CPE) for the determination of macrolide antibiotics is not tested until now.

Table 1. Voltammetric determination of ERY, EES, AZI, CLA and ROX by different working electrodes and methods

Analyte	Working electrode	Method	pH	Linear range (μg mL^{-1})	LOD (μg mL^{-1})	Ref.
ERY	[a]HMDE	[b]SW-AdSV	8.0	0.05-0.4	0.0006	28
ERY	HMDE	[c]AdSV	11.6	0.15-0.73	0.01	30
EES	HMDE	[d]LSPM	7.46	10-800	7.5	31
CLA	HMDE	[e]LS-AdCS [f]SW-AdCS	8.0	0.075-0.75 0.037-0.3	0.022 0.011	32
EES	Hg(Ag)FE	[g]SWV SW-AdSV	7.0	4.53-29.8 0.69-2.44	1.36 0.21	33
AZI	Hg(Ag)FE	SWV SW-AdSV	7.2	4.81-23.3 1.0-2.46	1.44 0.30	34
CLA	Hg(Ag)FE	SWV SW-AdSV	7.4	1.96-28.6 0.05-0.99	0.59 0.015	34
ROX	Hg(Ag)FE	SWV SW-AdSV	7.0	1.48-25.9 0.10-0.99	0.44 0.03	34
ERY	[h]GCE	second differential [i]ASV	9.0	0.018-0.26 (t_{acc}=90 s) 0.009-0.18 (t_{acc}=120 s)	0.004 (t_{acc}=360 s)	35
ERY	[j]AB/GCE	[k]DPV	7.5	0.15-7.3	0.06	36
AZI	GCE	DPV	7.0	1-15	0.7	38
AZI	$MgCr_2O_4$-[l]MWCNT/GCE	DPV	7.0	0.19-3.0 3.0-7.5	0.05	39
AZI	Polycrystalline-[m]AuE	[n]CV	8.48	235-588	Not reported	40
AZI	GCE	[o]DP-AdSV	6.0	1.0-10.0 (t_{acc}=60 s) 0.25-2.0 (t_{acc}=240 s)	0.29 (t_{acc}=60 s) 0.11 (t_{acc}=240 s)	42
AZI	CPE	SW-AdSV	4.6	0.000471–0.00707	0.000463	43
AZI	[p]MIP/AB/CPE	DPV	7.0	0.075-1.5 1.5-15.0	0.008	44
ROX	AuE	CV DPV	8.4	100-654 101-476	Not reported	45
ROX	Poly(3,4-ethylenedioxythiophene)-AuE	CV	7.0	0.067-16.74	0.022	46
ROX	[r]SWCNT/GCE	ASV	7.0	4.19-84	0.42	47

[a]HMDE – hanging mercury drop electrode, [b]SWV-AdSV – adsorptive stripping square wave voltammetry, [c]AdSV – adsorptive stripping voltammetry, [d]LSPM – linear scanning polarographic method, [e]LS-AdCS – linear-sweep adsorptive cathodic stripping voltammetry, [f]SW-AdCS – square-wave adsorptive cathodic stripping voltammetry, [g]SWV – square wave voltammetry, [h]GCE – glassy carbon electrode, [i]ASV – anodic stripping voltammetry, [j]AB – acetylene black nanoparticles, [k]DPV – differential pulse voltammetry, [l]MWCNT – multiwall carbon nanotubes,

[m]AuE – gold electrode, [n]CV – cyclic voltammetry, [o]DP-AdSV – adsorptive stripping differential pulse voltammetry, [p]MIP/AB – molecularly imprinted polymer/acetylene black, [r]SWCNT – single-walled carbon nanotubes.

In this work, results of investigation of voltammetric behavior of macrolide antibiotics EES, AZI, CLA and ROX at unmodified CPE in Britton-Robinson buffer solutions (from pH 2.0 to 11.98) by SWV are presented. Under the optimized experimental conditions the all investigated macrolide antibiotics were quantitatively determined in low μg mL^{-1} concentration ranges. Additionally, the applicability of AuNPs/CPE for direct anodic SWV determination of AZI and ROX was examined. The optimized experimental conditions with the use of SWV method as well as unmodified CPE and AuNPs/CPE were tested for determination of ROX and AZI in pharmaceutical preparations Runac® and Hemomycin®, respectively.

2. Experimental

2.1. Reagents and solutions

All chemicals used were of analytical reagent grade. The stock solutions of investigated macrolide antibiotics (100.0 μg mL^{-1}) were prepared individually by dissolving the appropriate amount of EES, azithromycin dihydrate, CLA and ROX in the mixture of double distilled water and methanol in the ratio 80%:20% (V/V) in the case of EES, CLA and ROX and 70%:30% (V/V) in the case of AZI, under ultrasound treatment.

The pharmaceutical preparations of selected macrolide antibiotics i.e. Hemomycin® and Runac® (Hemofarm, Vršac, Serbia) declared to contain 250 mg AZI per capsule and 150 mg ROX per tablet, respectively, were analyzed as real samples. The solutions of pharmaceutical preparations were prepared separately by dissolving of the appropriate mass of Hemomycin® capsule or Runac® tablet in the mixture of double distilled water and methanol in the ratio 70%:30% (V/V) and 80%:20% (V/V), respectively. For voltammetric and HPLC-DAD measurements obtained solutions of pharmaceutical preparations were filtered by 0.22 μm syringe filters (Kinesis, Reg. Cellulose) and further diluted as required.

Britton-Robinson buffer solutions as supporting electrolyte in pH range from 2.0 to 11.98 were prepared from the mixture of 0.04 mol L^{-1} boric, phosphoric and acetic acids by adding of appropriate amount of 0.2 mol L^{-1} sodium hydroxide.

2.2. Electrodes and Apparatus

Voltammetric measurements were performed at an electrochemical analyzer AUTOLAB PGSTAT12 operated via GPES 4.9 software using three-electrode system consisting of saturated calomel electrode (SCE) as reference, platinum as auxiliary and unmodified CPE or AuNPs/CPE as working electrode. All potentials referred in this work are given as values against the SCE.

pH values were measured with a digital pH meter (inoLab® Multi 9620 IDS, Germany) using a combined glass 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).

Morphology of CPE and AuNPs/CPE surfaces was investigated by scanning electron microscope (SEM, HITACHI S-4700 Type II cold field emission) while the presence of AuNPs on the electrode surface was confirmed by energy dispersive spectrometer (EDS, Röntec QX2). Spectra were analyzed by the factory standard software after automatic background subtraction and peak fitting.

2.3. Procedures

Preparation of working electrodes. The CPEs were prepared by hand mixing of 0.5 g graphite powder (< 20 μm, synthetic, Sigma-Aldrich) and 0.2 mL paraffin oil (Kemika, Zagreb, Croatia) and after proper homogenization, the obtained pastes were packed into piston-driven Teflon® holders with a diameter of 2 mm [73]. The electrode surface was renewed before each set of measurements mechanically by pushing out small amount of paste from holder and by wiping of electrode surface with piece of clean paper. Additionally, CPE was surface modified by drop coating method i.e. 3.0 μL of synthesized suspension of gold nanoparticles of 10 nm diameter was dropped onto renewed and prepared electrode surface and the resulting AuNPs/CPE was dried at room temperature ca. 45 minutes.

Synthesis of AuNPs was carried out according to the procedures first described as pioneering work in the citrate reduction method [74]. A model for the formation of gold nanoparticles in the citrate synthesis method with the overview of its development from its invention until now is presented in [75]. Briefly, 80 mL of 2 mg mL^{-1} sodium citrate dihydrate solution was heated to 80 °C. Once the temperature was reached, two further solutions were

added to the reaction. Firstly, 2 mL (1 mg mL^{-1}) of freshly prepared sodium borohydride and ultimately 3.2 mL of 10 mg mL^{-1} chloroauric acid under constant vigorous stirring. The resulting purple-red suspension was further stirred for 1 hour on 80 °C and then the sample was left to cool to room temperature at which point the stirring was stopped. The formed AuNPs solution was kept in dark until usage.

Conditions of SEM/EDS measurements. The surface morphology of unmodified CPE and AuNPs/CPE were investigated by SEM with an acceleration voltage of 10.0 and 20.0 kV while the selected surface areas were analyzed using EDS detector with 20.0 kV.

V*oltammetric measurements in model solutions and real sample analysis.* The voltammetric behavior of EES, AZI, CLA and ROX was investigated by SWV methods in Britton-Robinson buffer solutions as supporting electrolyte from pH 2.0 to pH 11.98 using unmodified CPE. In all voltammetric measurements, the solution of Britton-Robinson buffer was two times diluted with double-distilled water and the solutions without target analyte served as a blank. The measurement parameters in SWV were as follows: potential range from 0.4 to 1.1 V, the pulse amplitude 20 mV, step potential 5 mV, and frequency 50 Hz. In the case of AZI and ROX, the SWV measurements were performed with AuNPs/CPE, too. The reported signals were measured without subtracting the blank solution and using the integration procedure available in the GPES 4.9 software. In this procedure the baseline is determined by means of the tangent fit method, whereby the tangent is drawn from the left side to the right side of a peak. The limit of detection (LOD) and limit of quantitation (LOQ) were calculated as signal to noise of baseline ratio of three and ten, respectively.

The content of AZI and ROX in their pharmaceutical preparations was determined by standard addition method using developed SWV methods (pH 11.98) and CPE in the case of ROX, or the same pH of supporting electrolyte and AuNPs/CPE in the case of AZI. After addition of appropriate volume of filtered real sample into the voltammetric vessel, three standard additions of stock solutions of AZI or ROX were added. The final concentrations of standard solutions of AZI in voltammetric vessel were 0.58; 1.16 and 1.74 μg mL^{-1} while in the case of ROX, they were 0.97; 1.91 and 2.84 μg mL^{-1}. All measurements were performed in triplicates.

Comparative liquid chromatographic measurements. The conditions of reversed phase HPLC-DAD measurements were similar as in our previous work [33]: mobile phase mixture of

0.02 mol L^{-1} phosphate buffer pH 8.0 and acetonitrile (45%:55%, V/V), flow rate 0.3 mL min^{-1}, injected volume of sample 20 µL, column temperature 25 °C and working wavelength of the detector 215 nm with reference wavelength at 500 nm. After HPLC analysis, the calibration curves of ROX and AZI were constructed for both macrolide antibiotics by plotting peak area *vs* macrolide concentration in the range 5.0-50.0 µg mL^{-1}. The ROX and AZI content in their pharmaceutical preparations was determined from the area of the corresponding peaks and the calibration curves equations. All measurements were performed in triplicates.

3. Results and discussion

3.1. SEM characterization of working electrodes

Surface morphology and chemical composition of working electrodes, CPE (Fig. 2A) and AuNPs/CPE (Fig. 2B), were studied by SEM/EDS measurements. It can be seen that both surfaces contain relatively compact carbon paste matrix made of graphite particles and paraffin oil. Additionally, in the case of AuNPs/CPE, the bright particles which are randomly distributed on the carbon paste surface as independent units or in form of smaller aggregates can be recognized. These particles are dominantly with spherical shape of around 10 nm diameters and penetrate to the first layers of the paste with a similar distribution as on the surface. It should be mentioned that CPE surface was modified with previously optimized amount of AuNPs suspension i.e. 3.0 µL by very simple drop coating protocol and without addition of any adhesive demonstrating good affinity of nanoparticles to the basic carbon paste matrix. This was proven by immersing of AuNPs/CPE in Britton-Robinson buffer solution pH 11.98, and after its staying in such media for a while, the SEM measurements of the selected surface area were performed and it was clearly visible that bright particles were still on the electrode surface (Fig. 2C) and by EDS it was confirmed that these particles are AuNPs (Fig. 2D).

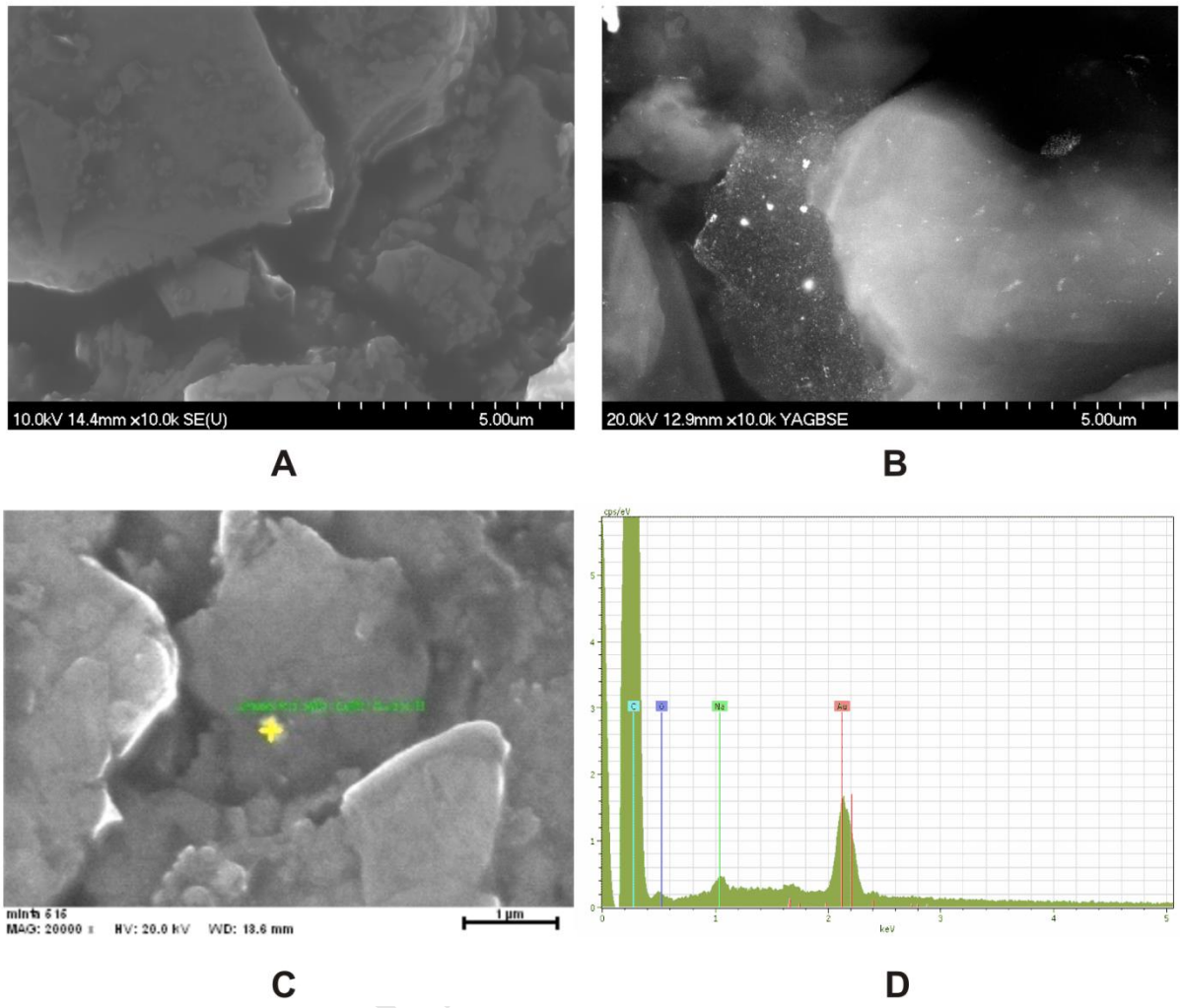

Fig. 2. Scanning electron micrographs of CPE (A) and AuNPs/CPE (B) surfaces obtained at the magnification of 10000x and representative part of the AuNPs/CPE surface (C) with appropriate EDS microanalysis spectra of the AuNPs (D). Detection mode: secondary electron imaging (A) and back scattered electrons (B).

3.2. Optimization of direct anodic SWV methods for selected macrolide antibiotics determination by CPE

In Fig. 3A (inset) are displayed cyclic voltammograms (CVs) of CPE (black curve) and AuNPs/CPE (red curve) in Britton-Robinson buffer pH 11.98. The same shape of the presented voltammograms is obtained by other authors working with this type of electrode surfaces [76, 77]. The CPE surface was modified with only 3.0 µL of colloidal gold suspension (100 µg mL^{-1})

and as can be seen from obtained SEM results the AuNPs are randomly distributed over the electrode surface and there are also modifier free regions meaning that CPE surface is not completely covered with AuNPs. Because of the low amount of the modifier, the carbon paste properties are prevalent, and the obtained CV for AuNPs/CPE has no the shape as it is characteristic for bare gold electrode and is similar with that obtained for unmodified CPE which is in accordance with previously published papers [76, 77]. Nevertheless, the electrocatalytic effect of AuNPs will be recognized in the presence of analytes leading to the higher intensity of the macrolides oxidation peaks.

Previous investigations of the reduction of macrolide antibiotics by Hg(Ag)FE showed that the pH value of the supporting electrolyte has great influence on the peak shape and peak intensity [33, 34]. Therefore, the first optimization step during development of direct anodic SWV methods for macrolide antibiotics determination was selection of optimal pH value. Accordingly, the unmodified CPE was applied in the pH range from 2.0 to 11.98 with the aim to study the influence of pH value on the peak shape and peak intensity of all investigated macrolides. SW voltammograms were recorded in the absence (Fig. 3A) and in the presence of appropriate concentrations of macrolides (Fig. 3B-E).

The first measurable oxidation peaks of macrolide antibiotics appeared at pH 6.0 and at higher pH values as it can be seen from the representative SW voltammograms presented in Fig. 3. Carbon paste is almost inert electrode material with substantial resistance against unwanted chemical or electrolytic transformations, but its surface can be oxidized under extreme conditions. Common types of CPE could be polarized up to 1.0 V *vs* SCE, afterwards the oxidation of electrode occurs [78]. At the potentials of analytes peaks appearing, the obtained anodic currents could be attributed only to the anodic reactions of macrolides.

In the case of EES (Fig. 3B) and AZI (Fig. 3C) only one well-defined oxidation peak can be recognized with peak maximums in the potential range from around 0.95 V to 0.75 V depending on pH value of the supporting electrolyte. In contrast, two other macrolides i.e. CLA (Fig. 3D) and ROX (Fig. 3E) at pH values higher than 8.0 and 9.0, respectively, give two oxidation peaks at investigated concentration level whereby the first peak which appeared at more negative potential values was more suitable for analytical purposes due to the higher intensity and better shape comparing to the second oxidation peak of target analytes.

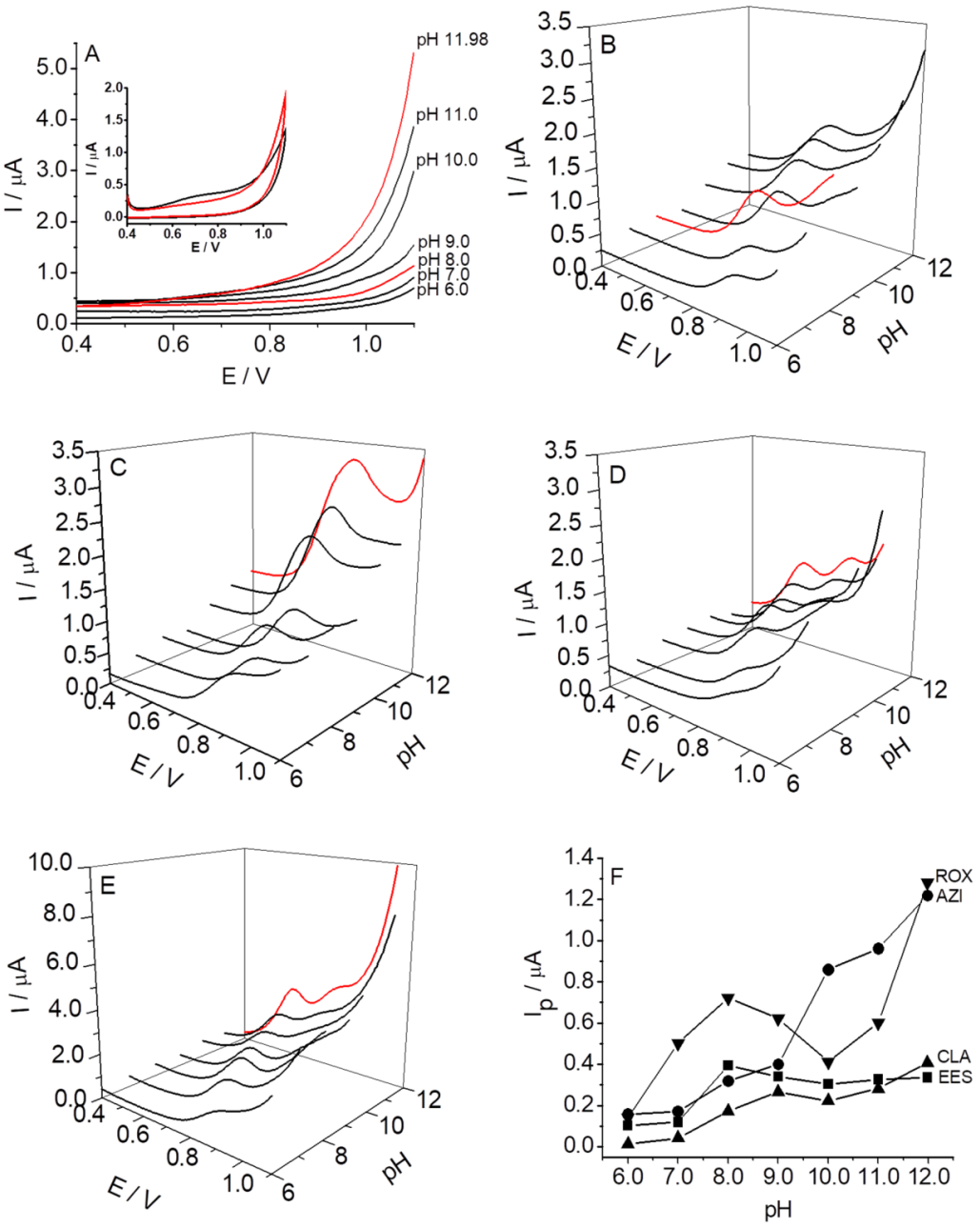

Fig. 3. CVs of CPE (black curve) and AuNPs/CPE (red curve) recorded in Britton-Robinson buffer pH 11.98 with scan rate 50 mV s^{-1} (inset, A). SW voltammograms of Britton-Robinson buffer solutions/blank samples (A), 27.1 µg mL^{-1} EES (B), 24.7 µg mL^{-1} AZI (C), 33.3 µg mL^{-1} CLA (D) and 33.3 µg mL^{-1} ROX (E) recorded by CPE in the pH range from 6.0 to 11.98. Appropriate I_p-pH dependences for investigated macrolide antibiotics (F). Red curves present SW voltammograms at optimal pH values.

Based on the obtained signals appropriate dependences of pH value on the peak potential (E_p, not shown) and peak intensity (I_p, Fig. 3F) were constructed for all investigated macrolide antibiotics and it was noted that pH value has strong influence on their peak shape and peak intensity using CPE as working electrode. In all cases, the oxidation peak potentials shift to the negative direction with the increasing pH of the supporting electrolyte and the obtained E_p-pH dependences could be described with linear equations and main parameters summarized in Table 2. In the case of EES and AZI two linear ranges of E_p-pH dependence were obtained, first from pH 6.0 to pH 8.0 and second from pH 8.0 to pH 11.98.

Table 2. Selected parameters of the obtained linear E_p-pH dependences by SWV and unmodified CPE for EES, AZI, CLA and ROX in the investigated pH range

Parameters	EES		AZI		CLA	ROX
	pH 6.0-8.0	pH 8.0-11.98	pH 6.0-8.0	pH 8.0-11.98	pH 6.0-11.98	pH 6.0-11.98
Intercept (V)	1.30	1.01	1.12	0.91	1.23	1.16
Slope (V/pH)	-0.058	-0.022	-0.035	-0.007	-0.051	-0.046
Correlation coefficient	-0.989	-0.994	-0.988	-0.996	-0.994	-0.989

As suggested in our previous papers [37, 40, 45] and the literature cited there, of the different functional groups present in macrolide antibiotics, the amine group is the most easily oxidizable one. Oxidation of dialkylamines is proceeding through a loss of an electron which leads to formation of a cation radical. The same can be adopted for macrolide antibiotics where 3^o alkyl group is present on the desosamine sugar radical. It is also probable that the demethylation reaction occurs, giving the corresponding secondary amine. Shortly, for the mechanism of the ROX oxidation, from different functional groups of ROX, the amine group is the most easily oxidizable. Dialkylamines are oxidized forming a radical cation by loss of electron. The similar voltammetric behavior was observed with ERY which indicated that the mechanism proposed for the anodic oxidation of ROX is initiated by one-electron transfer to form the cation radical at nitrogen on the desosamine sugar radical [45]. Erythromycin A underwent oxidative degradation on gold electrode, probably the first step in the oxidation process is the removal of the electron from one of the nitrogen atoms to form an aminium cation radical. Formed aminium radical cation is a very reactive species and rapidly reacts with the

environment to form stable products. The formed radical cation can abstract hydrogen atom from the water resulting in salt formation in an overall one-electron process. Hence, the formed cation inhibits further electrochemical oxidation [37].

Peak intensity, peak shape and reproducibility of analytical signal were the main criteria for selection of optimal pH value for analytical purposes. As can be seen from Fig. 3F the lowest peak intensity for all macrolides was obtained at pH 6.0. The most intensive peak of EES was obtained at pH 8.0 and at higher pH values the peak intensity slightly decreased. From pH 9.0 to 11.98 there is no big difference between I_p of obtained EES signals (Fig. 3B, F). So, based on the peak shape and peak intensity pH 8.0 was chosen as optimal pH value of supporting electrolyte for further determination of EES. In the case of AZI, the I_p increased from pH 6.0 to pH 11.98 around two times (Fig. 3C, F). The I_p of CLA increased from pH 6.0 till pH 9.0, at pH 10.0 signal decreased and then at higher pH values the peak intensity again increased (Fig. 3D, F). In the case of ROX the intensity of first oxidative signal increase to pH 8.0 and then start decreasing till pH 10.0 and as it is the case with CLA from pH 10.0 signal start increasing again (Fig. 3E, F). Based on the above, the appropriate shape and most intensive peaks of AZI, CLA and ROX were achieved at pH 11.98 and therefore the further voltammetric measurements of these macrolides were performed at mentioned pH value of supporting electrolyte.

In order to achieve the sensitive and reliable voltammetric methods for macrolide antibiotics determination, it must be mentioned that some additional measurements were performed. Namely, beside SWV, differential pulse voltammetric (DPV) measurements were also carried out, but the signals were with lower intensity compared to SW voltammetric ones. For example, the DPV oxidation signal of AZI was around three times lower than its SWV signal at the same concentration level, and there were obvious differences in their shape, too. So, the priority of SWV method was confirmed since the better peak shape and better sensitivity for all four macrolide antibiotics studied in this work were achieved.

Another investigated parameter was how the mechanical renewing or non-renewing of the CPE surface could affect the reproducibility of the analytical signal of macrolide antibiotics. This was studied for CLA by consecutively recording 10 direct anodic SWVs in the solution of target analyte (33.3 μg mL^{-1}) at pH 11.98, and based on the I_p of obtained signals RSD was calculated. With mechanical renewing of CPE surface before each measurement the RSD was 25.9% while the RSD was 8.7% when all measurements were performed with the same electrode

surface, indicating that the further determination of CLA by CPE should be performed with non-renewed electrode surface because at these conditions was achieved better reproducibility of the analytical signal. In order to additionally improve the RSD, it was examined how pre-cycling affect the reproducibility of the analytical signal. The cyclic voltammograms were recorded (20 cycles) in the potential span from 0.4 V to 1.1 V with a scan rate of 0.25 V s^{-1}, and then 10 consecutive SW voltammograms were recorded in the same solution of CLA (33.3 μg mL^{-1}) without renewing of the CPE surface. By this type of electrochemical pretreatment, RSD was decreased to 3.3%. It can be concluded that the previous cycling of the electrode and the non-renewing of the CPE surface during determination of CLA lead to more stable signal of the target analyte. Also, it was shown that the optimization of different measurement steps is very important and must be carefully carried out to achieve the best conditions for determination of target analytes i.e. macrolide antibiotics in present case.

3.3. SWV determination of selected macrolide antibiotics in model solution by CPE and AuNPs/CPE

Direct anodic SWV methods for macrolide antibiotics determination were further developed using CPE as working electrode and optimal pH values of the supporting electrolyte. SWVs were recorded individually for EES, AZI, CLA and ROX in appropriate concentration ranges and corresponding calibration curves were constructed. From there evaluated analytical parameters are presented in Table 3. It can be seen that each macrolide antibiotic has characteristic behavior at CPE, whereby the one linear range was obtained for AZI and two linear ranges for EES, CLA and ROX in investigated concentration ranges with correlation coefficient of calibration curves equal to or higher than 0.991. It can be assumed that the first linear range can be applied for the determination of investigated macrolides in real samples using standard addition method. The most sensitive SWV method using CPE was achieved for AZI while the highest LOQ was observed for CLA. As an example, in Fig. 4A are presented SWV curves recorded for ROX in concentration range 0.99-23.1 μg mL^{-1} together with appropriate calibration curve which was described by two linear ranges (inset). It can be noted that the second oxidation peak of ROX appeared from concentration of 6.54 μg mL^{-1}, but there were no significantly changes in its intensity with changing of concentration of target analyte and it has much lower intensity comparing with first peak, so for analytical purposes first oxidation peak of

ROX was analyzed. To ensure the reliability of developed SWV methods, investigation of analytical signals reproducibility is very important task. So, for each macrolide antibiotic RSDs were calculated based on the six consecutively recorded SWVs at selected concentration levels to cover both linear ranges and obtained values did not exceed 6%. In Fig. 4B are presented six consecutive recorded SW voltammograms in the presence of 4.76 μg mL^{-1} of ROX and calculated RSD was 1.3%, while in the case of higher concentration as 13.8 μg mL^{-1} RSD was 1.9%.

Table 3. Selected analytical parameters of four macrolide antibiotics determination in model solutions by unmodified CPE at pH 8.0 (EES) and pH 11.98 (AZI, CLA, ROX).

Parameters	EES		AZI	CLA		ROX	
	I linear range	II linear range		I linear range	II linear range	I linear range	II linear range
Linear range (μg mL^{-1})	0.59-5.60	5.60-14.4	0.15-2.34	4.76-7.41	7.41-37.5	0.99-6.54	6.54-23.1
Intercept (μA)	0.003	0.067	-0.0002	0.004	0.096	0.021	0.200
Slope (μA mL $μg^{-1}$)	0.016	0.004	0.158	0.022	0.008	0.060	0.029
Correlation coefficient	0.996	0.991	0.999	0.994	0.998	0.996	0.999
LOD (μg mL^{-1})	0.18	-	0.045	1.43	-	0.30	-
LOQ (μg mL^{-1})	0.59	-	0.15	4.76	-	0.99	-

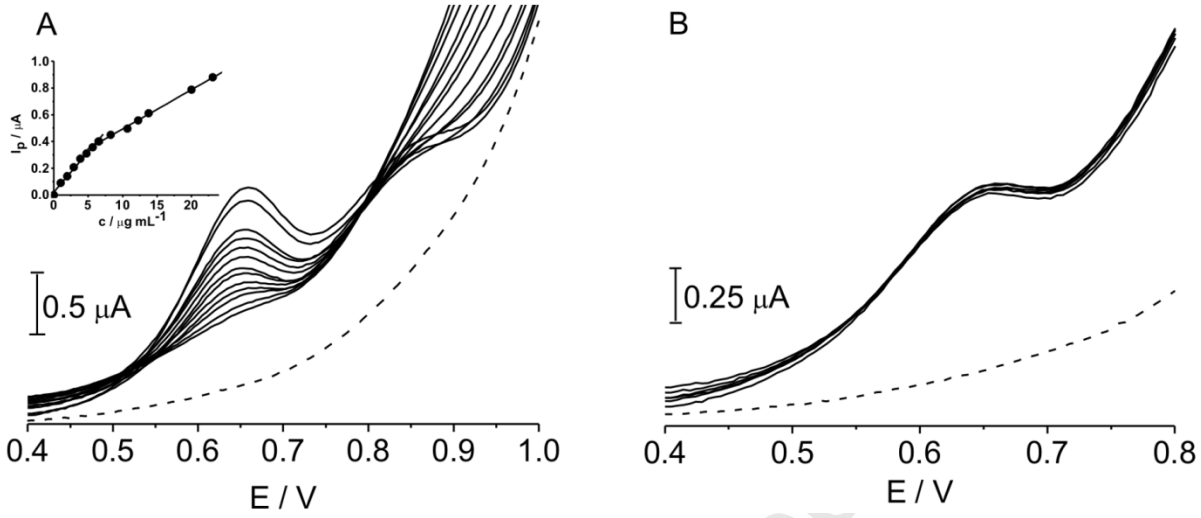

Fig. 4. SW voltammograms of ROX obtained by CPE in concentration range from 0.99 to 23.1 μg mL^{-1} (A) and appropriate calibration curve (inset). Reproducibility of the analytical signal (six repetitions) for 4.76 μg mL^{-1} ROX (B). Dashed lines illustrate the appropriate baseline at pH 11.98.

Considering the received results, it was demonstrated that very simple direct anodic SWV methods in combination with easy-to-prepare CP working electrode could be applied for determination of different target analytes from the group of macrolide antibiotics. However, investigations were continued to determine whether the incorporation of AuNPs to the surface of CPE can improve the analytical performance of previously described unmodified CPE. The SWV measurements were carried out with AZI (Fig. 5A) and ROX as target compounds using AuNPs/CPE in the Britton-Robinson buffer pH 11.98 as supporting electrolyte, since this was the optimal pH value for their determination in model solutions.

At the potentials of the analytes oxidation reaction appearing (Fig. 5), at bare gold electrode proceeds the oxide formation, necessary for the oxidation of the all macrolides [37, 40, 41, 45, 79-81]. The cyclic voltammetry of gold electrode and oxide formation in alkaline electrolytes is described in detail in [82] showing that examined macrolides undergo to their oxidation as was already published for bicarbonate electrolyte (pH 8.4), in the area of oxide formation. The anodic peaks appearance due to the macrolides oxidation proceeds also in the area of the beginning of the oxide formation at the electrode surface.

In the Table 4 are summarized the basic analytical parameters of SWV methods for both analytes. In the case of AZI, although the same LOD value was obtained with CPE and

AuNPs/CPE, the modified electrode was the priority one because of the wider linear range of calibration curve (Fig. 5A, inset) and better reproducibility of the analytical signal. Namely, based on the six repeated SWV measurements of 0.99 μg mL^{-1} AZI (Fig. 5B) using AuNPs/CPE the RSD was 3.4%, and it was almost two times lower than the value obtained with unmodified CPE. On the other hand, from the data related to the determination of ROX presented in Table 3 and 4, it can be said that except wider linear range, the sensitivity of the SWV method was also improved by AuNPs/CPE leading to the two times lower LOD value for ROX compared to one achieved by CPE. The reproducibility of ROX signal was investigated as well, and calculated RSD for 3.85 μg mL^{-1} concentration level of ROX was 0.75%. Therefore, it can be concluded, that presence of optimized amount of the AuNPs on the CPE surface has positive influence on AZI and ROX oxidation signals probably due to the pronounced conductivity of electrode surface and electrocatalytic properties of AuNPs.

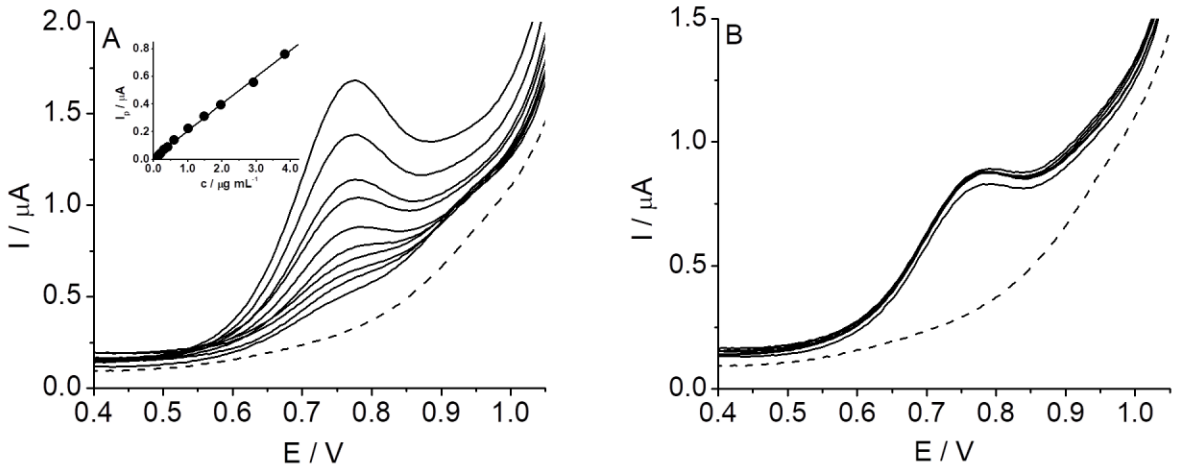

Fig. 5. SW voltammograms of AZI obtained by AuNPs/CPE in concentration range from 0.15 to 3.84 μg mL^{-1} with appropriate calibration curve in inset (A) and reproducibility of the analytical signal (six repetitions) for 0.99 μg mL^{-1} AZI (B). Dashed lines illustrate the appropriate baselines at pH 11.98.

It must be mentioned that obtained LODs and LOQs of developed direct SWV methods for determination of macrolide antibiotics presented in this work are lower or in the same range as mostly of previously reported ones for reduction or oxidation of these target analytes (see Table 1). Besides, herein described methods are based on the use of simple but contemporary

working electrodes i.e. CPE or AuNPs/CPE, which are environmental friendly and economically acceptable since the very small amount of modifier on CPE surface contribute to the overall improvement of its analytical performance.

Table 4. Selected analytical parameters of AZI and ROX determination in model solutions by AuNPs/CPE at pH 11.98.

Parameters	AZI	ROX	
		I linear range	II linear range
Linear range (μg mL^{-1})	0.15-3.84	0.50-9.09	9.09-37.5
Intercept (μA)	0.013	0.019	0.488
Slope (μA mL μg^{-1})	0.194	0.126	0.069
Correlation coefficient	0.999	0.998	0.998
LOD (μg mL^{-1})	0.045	0.15	-
LOQ (μg mL^{-1})	0.15	0.50	-

3.4. SWV determination of selected macrolide antibiotics in appropriate pharmaceutical preparation

The quality control of two different pharmaceutical preparations named Runac® and Hemomycin® was performed by optimized SWV methods including CPE or AuNPS/CPE as working electrode to determine their active ingredient content. Simple preparation procedure consisted only of filtering tablets/capsule suspensions by hydrophilic syringe filters 0.22 μm before the measurement. In both cases standard addition method was applied to avoid possible influence of the matrix, since the selected pharmaceutical preparations also contain some additional components except their active ingredients ROX or AZI.

Determination of ROX in its pharmaceutical preparation Runac®

The practical applicability of optimized SWV method in combination with unmodified CPE was tested for determination of ROX in pharmaceutical preparation Runac® (declared content of ROX by the producer: 150 mg/tablet). The measurements were performed in concentration range which belongs to the first linear range of calibration curve (Fig. 6). After

recording the voltammogram of blank sample/baseline (Fig. 6A, curve 1, dashed line), the appropriate aliquot of tablet solution was added into supporting electrolyte pH 11.98 (Fig. 6A, curve 2) followed by three standard additions of ROX (Fig. 6A, curves 3-5) with their final concentrations in voltammetric vessel as 0.97; 1.91 and 2.84 μg mL^{-1}. Found concentration of ROX was obtained from the corresponding analytical curve and in presented case (Fig. 6B) it was 3.67 μg mL^{-1} and calculated to amount of ROX per one tablet: 150.5 mg/tablet. Two more repetitions were performed and the obtained values for ROX content in tablet were 151.3 and 150.9 mg/tablet. The average value from triplicate measurements was 150.9 mg/tablet which is very close to the content of ROX found by comparative HPLC-DAD measurements (average value 151.1 mg/tablet, RSD=2.65%) and satisfies the basic requirements of the European Pharmacopoeia 8 [83] in terms of errors in accuracy of the measurement. The calculated RSD for the SWV method was 0.15% indicating good repeatability and reliability of proposed SWV method.

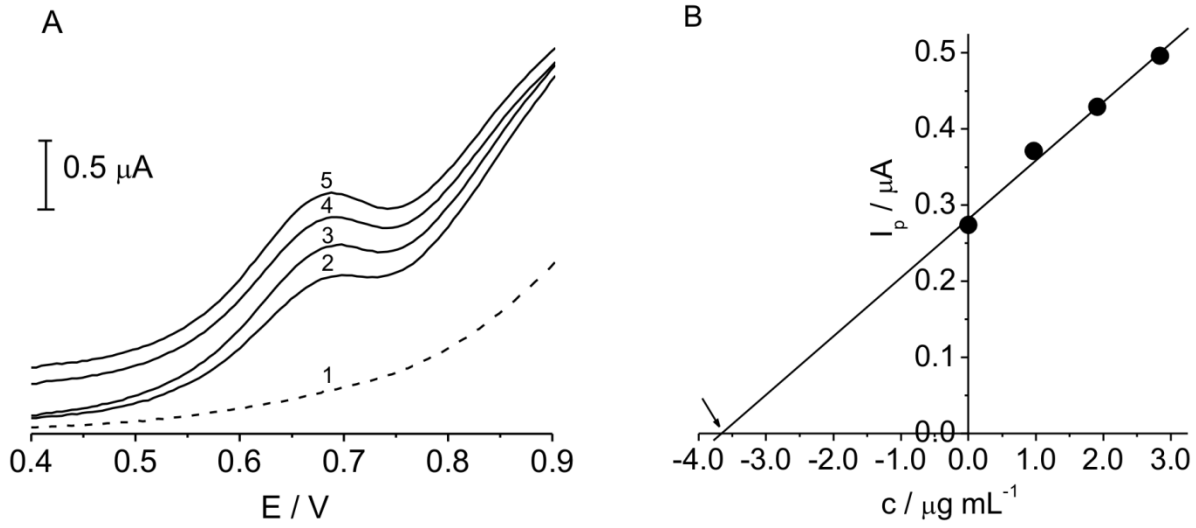

Fig. 6. Determination of ROX concentration in pharmaceutical preparation Runac® applying standard addition method and CPE at pH 11.98. SW voltammograms (A) of: baseline (1), sample of pharmaceutical preparation (2) and three standard additions of ROX (3-5, the final concentrations in voltammetric vessel: 0.97; 1.91 and 2.84 μg mL^{-1}) and appropriate analytical curve (B).

Determination of AZI in its pharmaceutical preparation Hemomycin®

The optimized SWV method and AuNPs/CPE were applied for determination of AZI in its pharmaceutical preparation Hemomycin® at pH 11.98 (Fig. 7) similarly as in the case of determination of ROX content in Runac®. In Fig. 7A are presented illustrative voltammograms obtained for the blank sample/baseline (curve 1, dashed line), sample of Hemomycin® (curve 2) and consecutively added three standard additions of AZI (curves 3-5). The concentration of AZI was evaluated from the analytical curve (Fig. 7B) and expressed as mg of AZI per capsule. The measurements were performed in triplicates, whereby the results obtained for the AZI content in the sample of the Hemomycin® capsule were as follows: 247.2 mg, 250.0 mg and 248.6 mg (average value 248.6 mg/capsule), with RSD of 0.6% for SWV method. Also, it should be noted that these triplicate SWV measurements were performed with the same electrode surface, demonstrating the good stability of the prepared AuNPs/CPE sensor. On the other hand, in order to check the reproducibility of modified electrode preparation, SWV measurements of AZI in its pharmaceutical preparation were also carried out according to previously described procedure, but by three times independently prepared AuNPs/CPE surfaces with the same drop coating protocol. The obtained average value in this case was 251.4 mg of AZI/capsule with RSD of 0.6%, which proved that the surface modification of CPE with AuNPs is performed on very reproducible way. The found amount of AZI in Hemomycin® capsule by proposed SWV method and AuNPs/CPE was in a good agreement with the amount found by comparative HPLC-DAD measurements (average value 254.1 mg/capsule, RSD=0.7%) and the value declared by the producer (250 mg/capsule).

Based on the above, CPE and AuNPs/CPE were found to be excellent analytical tools for the analysis of pharmaceutical preparations with different macrolide antibiotics as active ingredients. Although the comparative 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 lower LOD and LOQ values. Further investigations are planned for designing of similar types of carbon paste based working electrodes and widening of their applications concerning the determination of other important physiologically active compounds.

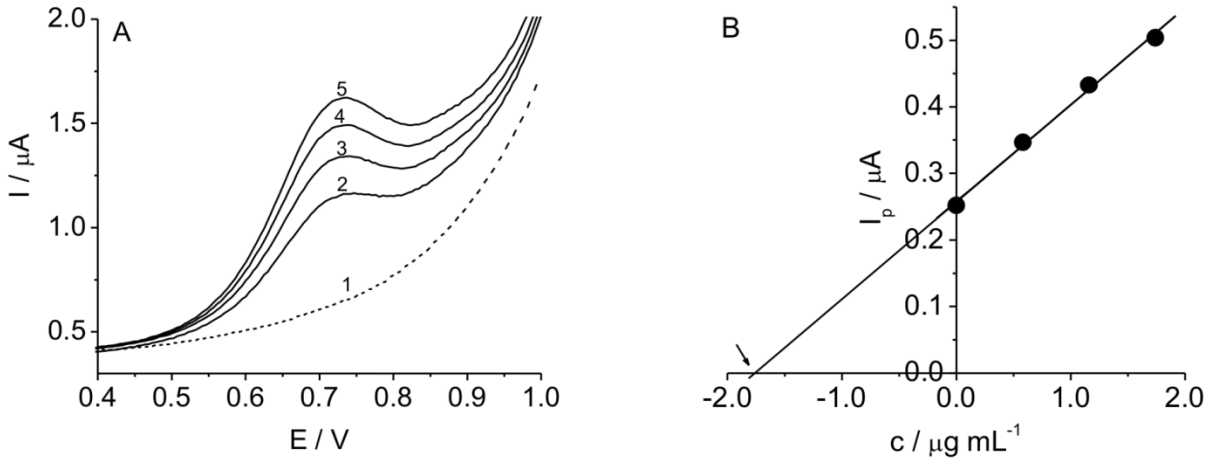

Fig. 7. Determination of AZI concentration in pharmaceutical preparation Hemomycin® applying standard addition method and AuNPs/CPE at pH 11.98. SW voltammograms (A) of: baseline (1), sample of pharmaceutical preparation (2) and three standard additions of AZI (3-5, the final concentrations in voltammetric vessel: 0.58; 1.16 and 1.74 μg mL^{-1}) and appropriate analytical curve (B).

4. Conclusion

CPE which was made only from graphite powder and paraffin oil proved to be suitable as working electrode for voltammetric determination of selected macrolide antibiotics as EES, AZI, CLA and ROX based on their oxidation. The direct anodic SWV methods were developed and optimization of the pH of supporting electrolyte showed that the alkaline media is favorable for macrolide antibiotics determination considering the peak shape and intensity. Applying direct anodic SWV method two linear ranges of calibration curve for EES, CLA and ROX, and one linear range for AZI were obtained with LOQs in the low μg mL^{-1} concentration range, which enable determination of selected macrolide antibiotics in their pharmaceutical preparations. On the other hand, since the achieved LOQ values are at concentration levels that are usually expected in biological fluids after administration of these antibiotics, there is possibility to determine them in such type of samples as well. The presented analytical methods are very simple, low-cost, and characterized by fast response time, good reproducibility and reliability.

Additionally, with the incorporation of small amount of AuNPs onto the CPE surface by drop coating method, it was possible to improve the sensitivity and reproducibility, and also expand the linear range of the methods in the case of two investigated macrolides AZI and ROX. It was demonstrated that by the optimized experimental conditions, the SWV method using CPE or AuNPs/CPE, was applicable for the determination of ROX and AZI in their pharmaceutical preparations Runac® and Hemomycin®, respectively, in terms of quick and reliable quality control indicating a good analytical performance of designed working electrodes and giving the possibility for further expanding of their application in pharmaceutical analysis.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Table 1. Voltammetric determination of ERY, EES, AZI, CLA and ROX by different working electrodes and methods

Analyte	Working electrode	Method	pH	Linear range (μg mL^{-1})	LOD (μg mL^{-1})	Ref.
ERY	[a]HMDE	[b]SW-AdSV	8.0	0.05-0.4	0.0006	28
ERY	HMDE	[c]AdSV	11.6	0.15-0.73	0.01	30
EES	HMDE	[d]LSPM	7.46	10-800	7.5	31
CLA	HMDE	[e]LS-AdCS [f]SW-AdCS	8.0	0.075-0.75 0.037-0.3	0.022 0.011	32
EES	Hg(Ag)FE	[g]SWV SW-AdSV	7.0	4.53-29.8 0.69-2.44	1.36 0.21	33
AZI	Hg(Ag)FE	SWV SW-AdSV	7.2	4.81-23.3 1.0-2.46	1.44 0.30	34
CLA	Hg(Ag)FE	SWV SW-AdSV	7.4	1.96-28.6 0.05-0.99	0.59 0.015	34
ROX	Hg(Ag)FE	SWV SW-AdSV	7.0	1.48-25.9 0.10-0.99	0.44 0.03	34
ERY	[h]GCE	second differential [i]ASV	9.0	0.018-0.26 (t_{acc}=90 s) 0.009-0.18 (t_{acc}=120 s)	0.004 (t_{acc}=360 s)	35
ERY	[j]AB/GCE	[k]DPV	7.5	0.15-7.3	0.06	36
AZI	GCE	DPV	7.0	1-15	0.7	38

AZI	$MgCr_2O_4$-[l]MWCNT/GCE	DPV	7.0	0.19-3.0 3.0-7.5	0.05	39
AZI	Polycrystalline-[m]AuE	[n]CV	8.48	235-588	Not reported	40
AZI	GCE	[o]DP-AdSV	6.0	1.0-10.0 (t_{acc}=60 s) 0.25-2.0 (t_{acc}=240 s)	0.29 (t_{acc}=60 s) 0.11 (t_{acc}=240 s)	42
AZI	CPE	SW-AdSV	4.6	0.000471–0.00707	0.000463	43
AZI	[p]MIP/AB/CPE	DPV	7.0	0.075-1.5 1.5-15.0	0.008	44
ROX	AuE	CV DPV	8.4	100-654 101-476	Not reported	45
ROX	Poly(3,4-ethylenedioxythiophene)-AuE	CV	7.0	0.067-16.74	0.022	46
ROX	[r]SWCNT/GCE	ASV	7.0	4.19-84	0.42	47

[a]HMDE – hanging mercury drop electrode, [b]SWV-AdSV – adsorptive stripping square wave voltammetry, [c]AdSV – adsorptive stripping voltammetry, [d]LSPM – linear scanning polarographic method, [e]LS-AdCS – linear-sweep adsorptive cathodic stripping voltammetry, [f]SW-AdCS – square-wave adsorptive cathodic stripping voltammetry, [g]SWV – square wave voltammetry, [h]GCE – glassy carbon electrode, [i]ASV – anodic stripping voltammetry, [j]AB – acetylene black nanoparticles, [k]DPV – differential pulse voltammetry, [l]MWCNT – multiwall carbon nanotubes, [m]AuE – gold electrode, [n]CV – cyclic voltammetry, [o]DP-AdSV – adsorptive stripping differential pulse voltammetry, [p]MIP/AB – molecularly imprinted polymer/acetylene black, [r]SWCNT – single-walled carbon nanotubes.

Table 2. Selected parameters of the obtained linear E_p-pH dependences by SWV and unmodified CPE for EES, AZI, CLA and ROX in the investigated pH range

	EES		AZI		CLA	ROX
Parameters	pH 6.0-8.0	pH 8.0-11.98	pH 6.0-8.0	pH 8.0-11.98	pH 6.0-11.98	pH 6.0-11.98
Intercept (V)	1.30	1.01	1.12	0.91	1.23	1.16
Slope (V/pH)	-0.058	-0.022	-0.035	-0.007	-0.051	-0.046
Correlation coefficient	-0.989	-0.994	-0.988	-0.996	-0.994	-0.989

Table 3. Selected analytical parameters of four macrolide antibiotics determination in model solutions by unmodified CPE at pH 8.0 (EES) and pH 11.98 (AZI, CLA, ROX).

Parameters	EES		AZI	CLA		ROX	
	I linear range	II linear range		I linear range	II linear range	I linear range	II linear range
Linear range (µg mL^{-1})	0.59-5.60	5.60-14.4	0.15-2.34	4.76-7.41	7.41-37.5	0.99-6.54	6.54-23.1
Intercept (µA)	0.003	0.067	-0.0002	0.004	0.096	0.021	0.200
Slope (µA mL $µg^{-1}$)	0.016	0.004	0.158	0.022	0.008	0.060	0.029
Correlation coefficient	0.996	0.991	0.999	0.994	0.998	0.996	0.999
LOD (µg mL^{-1})	0.18	-	0.045	1.43	-	0.30	-
LOQ (µg mL^{-1})	0.59	-	0.15	4.76	-	0.99	-

Table 4. Selected analytical parameters of AZI and ROX determination in model solutions by AuNPs/CPE at pH 11.98.

Parameters	AZI	ROX	
		I linear range	II linear range
Linear range (µg mL^{-1})	0.15-3.84	0.50-9.09	9.09-37.5
Intercept (µA)	0.013	0.019	0.488
Slope (µA mL $µg^{-1}$)	0.194	0.126	0.069
Correlation coefficient	0.999	0.998	0.998
LOD (µg mL^{-1})	0.045	0.15	-
LOQ (µg mL^{-1})	0.15	0.50	-

Graphical abstract:

Highlights:

- SWV determination of four macrolide antibiotics on CPE
- Optimized parameters employed on its modification AuNPs/CPE
- The morphology of AuNPs/CPE was characterized by SEM
- First time, AuNPs/CPE was tested in electro analytics of macrolides

- A good analytical performance of designed working electrodes