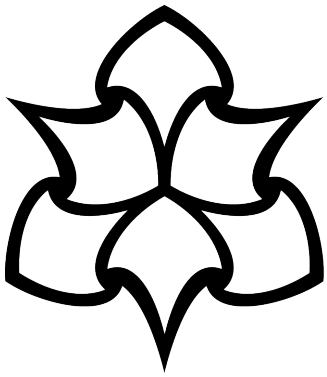

Application of botryosphaeran as a carbon black adherent on a glassy carbon electrode for the electrochemical determination of cyclobenzaprine

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Abstract

The present work describes the performance of a new voltammetric sensor based on the modification of glassy carbon electrodes (GCE) with carbon black (CB) and botryosphaeran (BOT) (CB-BOT/GCE) for the electroanalytical determination of cyclobenzaprine. BOT is a fungal exocellular (1→3)(1→6)-β-D-glucan, which was used to improve the adherence of CB onto the surface of GCE. The electrochemical characterisation was performed by electrochemical impedance spectroscopy which showed an improvement in the transfer of electrons on the surface of the sensor developed in relation to the unmodified (bare) GCE. The voltammetric behaviour of cyclobenzaprine was studied using bare GCE, BOT/GCE, CB/GCE, and CB-BOT/GCE. All electrodes presented an oxidation peak (+ 1.0 V) for cyclobenzaprine, while the cyclobenzaprine peak intensity on CB-BOT/GCE was found to be 480% higher than the bare GCE. Through employing square-wave voltammetry, the analytical curve was found to be linear over the concentration range of 2.0 to 20.6 μmol L^{-1} (in 0.1 mol L^{-1} NaCl solution) with a detection limit (based on 3-sigma) of 0.63 μmol L^{-1}. The developed electrochemical sensor exhibited excellent sensitivity and selectivity and was successfully applied for the voltammetric determination of cyclobenzaprine in pharmaceutical, biological, and environmental samples for the first time using the CB-BOT/GCE electrochemical sensing platform.

1. Introduction

Cyclobenzaprine hydrochloride (3-(5H-dibenzo[a,d]cyclohepten-5-ylidene)-N,N-dimethyl-1-propanamine hydrochloride), a centrally skeletal muscle relaxant, is widely used to relieve muscle spasms and pain associated with acute musculo-skeletal conditions, as well as for the treatment of fibromyalgia (myalgic encephalomyelitis/chronic fatigue) syndrome, and post-traumatic stress disorder [1,2]. This drug is a tricyclic amine that inhibits the presynaptic neuronal re-uptake of noradrenaline and serotonin that result in the immediate relaxation of muscle. Because of this effect, cyclobenzaprine is extensively used to treat muscular conditions, although in the case of overdose, it can cause cardiac arrhythmia, hallucinations, disorientation and abnormal behaviour, as well as dry skin and dry mouth-feel [2]. As a drug for human consumption, pharmaceuticals undergo several qualitative and quantitative pharmacopeia tests that assure safety and efficacy of treatment for the patients. It is important therefore that levels of cyclobenzaprine can be monitored in the patients' blood for clinic control purposes. In this context, it is necessary to develop a selective, sensitive, simple, and rapid method for the quantification of this drug.

Gas and liquid chromatography [[3], [4], [5], [6]–7] and spectrophotometry [8] procedures have been described for the determination of cyclobenzaprine, but require extensive sample preparation, the use of organic solvents, long analysis times, and relatively expensive instrumentation. In this context, electrochemical techniques based upon potentiometry and voltammetry have been depicted as alternatives for the quantification of organic molecules [[9], [10], [11], [12]–13]. These are simple, rapid, sensitive, selective, environmentally friendly, and relatively low-cost methods.

In this sense, a potentiometric sensor was developed by Ramadan et al. using microsized graphite for the determination of cyclobenzaprine. The sensor presented a linear dependence of potential within the concentration range of 10–10,000 μmol L^{-1}, a limit of detection (LOD) of 6.50 μmol L^{-1}, and was applied for the determination of cyclobenzaprine present in powder forms, tablets and blood serum samples [14]. However cyclobenzaprine determination in serum samples requires lower levels to be determine lower than that reported in Ref. [14]. Voltammetry, a very sensitive technique, has proved to be extremely suitable for the analysis of pharmaceuticals, biological fluids, and even environmental samples [15]. Despite this, no voltammetric methods have been described for the determination of cyclobenzaprine, and this is the main aim we report herein. Rodrigues and collaborates did an electrochemical characterization of cyclobenzaprine on the surface of carbon paste electrode, but they did not apply the electrode for the voltammetry determination of cyclobenzaprine in real samples [16]. A new voltammetric sensor using botryosphaeran (BOT) and carbon black (CB) is proposed for cyclobenzaprine determination. Polysaccharides have been applied for the development of new electrochemistry platforms, because they are non-toxic, biodegradable and adhere to solid surfaces [17,18]. BOT, first described by Barbosa et al., is an exopolysaccharide produced by the ascomyceteous fungus Botryosphaeria rhodina MAMB-05, and was structurally characterized as a (1→3)(1→6)-β-D-glucan [19]. The biological functions of BOT have recently been reviewed [20].

The electrochemical properties of BOT have also been explored in the construction of biosensors [21,22], in which BOT was used to immobilize the laccase enzyme. Although, BOT has not previous

been used to improve the adherence of CB on the surface of glassy carbon electrode (GCE) for the construction of an electrochemical sensor, it is this reason we utilized BOT in our work.

CB is a nanomaterial composed of sp2- and sp3-hybridized carbons, which has interesting physical characteristics for the field of electrochemistry, which include: large surface area, excellent conductivity, and physical and chemical stability [23]. Furthermore, CB is relatively low-cost when compared to others carbonaceous materials commonly used for the development of new electrochemical sensors [24,25]. Therefore, CB has been employed as a conductive/electrocatalytic material in the construction of (bio) sensors for the determination of a wide spectrum of analytes that have included, for example, organophosphorus compounds [26,27], phenolics [21,28,29], pesticides [24] and pharmaceuticals [[30], [31]–32].

Inspired by the above insights, we combined the characteristics of the carbohydrate biopolymer BOT and the carbonaceous material CB to develop a new electrochemical sensing platform. The sensor construction is based upon the modification of a GCE with CB and BOT (denoted as: CB-BOT/GCE), and its application for the electroanalytical determination of cyclobenzaprine in pharmaceutical, rat blood serum, and doped samples of river and tap water. This is the first work in the literature that presents a voltammetric determination of cyclobenzaprine.

2. Experimental

2.1. Reagents and solutions

All chemicals used in this study were of analytical grade. Solutions were prepared with ultra-purified water supplied by a Milli-Q system (Millipore®; resistivity > 18 MΩ cm). The cyclobenzaprine and K3[Fe(CN)6] were obtained from Sigma-Aldrich (St Louis, MO, USA), dimethylformamide (DMF) from Synth (99.8%, Diadema, SP, Brazil), and KCl, NaCl, BaCl, K2SO4, Na2SO4 were obtained from Anidrol (Diadema, SP, Brazil). GCE is from Tokay Carbon Co. (5 mm diameter; Tokay Carbon Co., Japan). Carbon black (CB; VXC72R) was kindly provided by Cabot Corporation (São Paulo, SP, Brazil). Pharmaceutical samples were obtained from a local pharmacy, in the city of Londrina, Paraná, Brazil.

Blood serum was obtained from a rat at Universidade Estadual de Londrina. The rat was left in a climatized room with a light/dark cycle of 12 h, and fed rat ration (Nuvital, Curitiba, PR, Brazil) and water ad libitum. At age 12 weeks, the rat was anesthetized (sodium thiopental, 40 mg Kg^{-1}), and a blood sample was taken from the abdominal aorta. The blood sample was centrifuged for 10 min at 1107 g and 4 °C. Serum was collected and stored at −80 °C. All the experimental protocols were approved for the Animal Research Ethics Committee of Universidade Estadual de Londrina (CEUA/UEL, nº 80/2017).

Stock solutions of cyclobenzaprine of 0.01 mol L^{-1} were freshly prepared by dissolving the standard in the supporting electrolyte (0.1 mol L^{-1} NaCl). Working solutions were prepared from the stock solution by appropriate dilutions. NaCl solution (0.1 mol L−1) used as supporting electrolyte was prepared by dissolving the salt in ultra-pure water.

2.2. Apparatus

All voltammetric measurements were carried out using an Autolab PGSTAT101 potentiostat/galvanostat (Metrohm Autolab B. V., The Netherlands) controlled by NOVA 2.1 software, and was coupled to a conventional three-electrode single-compartment glass cell. The working electrode used was CB-BOT/GCE, Ag/AgCl (3.0 mol L−1 KCl) as the reference electrode, and a platinum plate as the counter electrode.

Electrochemical impedance spectroscopy (EIS) measurements were performed in a FRAII μAUTOLAB type III potentiostat/galvanostat (Metrohm Autolab B. V., The Netherlands) controlled by NOVA 1.0 software. The EIS experiments were performed in the range of 10 mHz to 100 kHz (10 points per decade), and with a 10 mV (r.m.s.) ac perturbation.

An ultrasonic bath (Eco-Sonics Q3.8/37A, Indaiatuba, SP, Brazil) was used to prepare the CB dispersion in DMF for further modification of GCE in BOT film. For pH measurements, a Bel Engineering pH metre W3B (BEL, Monza, MB, Italy) was used, employing a glass electrode with an Ag/AgCl (3.0 mol L−1 KCl) external reference electrode.

The morphological characterisation of the different surfaces of the sensor was performed utilizing a scanning electron microscope (SEM; JOEL FEG-SEM JSM 6330F (JOEL, Peabody, MA, USA) operated at 5 kV. Samples for SEM were prepared by drop-casting the CB dispersion and BOT solution onto a glassy carbon surface.

2.3. Preparation of botryosphaeran

Botryosphaeran (BOT) was obtained according to the procedure elaborated by Barbosa et al. [19]. BOT was isolated from the cell-free fermentation broth of Botryosphaeria rhodina MAMB-05 following submerged fermentation on media containing 6% sucrose. BOT was precipitated from the cell-free extracellular fluid by adding 3 vol. ethanol and allowing the mixture to stand overnight at 4 °C. The precipitate (BOT) was recovered by centrifugation (1250 g/15 min), dissolved in deionized water, and then exhaustively dialyzed against distilled water for 48 h, with the distilled water changed every 12 h. The recovered solution following dialysis was lyophilized and stored at −2 ºC until required. A solution of BOT (0.78 mg mL−1) in ultra-pure water was used in the construction of the electrochemical sensor device (see below). Its concentration was determined by the phenol-sulfuric acid method [33].

2.4. Preparation of the CB-BOT/GCE

Initially, CB was dispersed in DMF, which is a better approach to disperse CB in DMF than water [34]. A dispersion of 2 mg CB in 1 mL of DMF was prepared in an ultra-sonic bath for 1 h at room temperature. The proportion between CB and DMF was examined in Section 3.2.1. For the modification of the electrode, the GCE (Ø = 3.0 mm) was polished with 0.5 µm alumina powder to obtain a mirror-like surface on the electrode and was then washed with ultrapure water every day before the modification steps. An aliquot of 3 µL of BOT solution and 3 µL of CB dispersion in DMF were cast dropwise simultaneously onto the GCE surface, and the solvent was allowed to evaporate for 3 h at room temperature. The steps in the fabrication of the electrochemical sensor device are shown in schematic form in Fig. S1 (Supplementary Material).

2.5. Analytical procedures

The electrochemical characterisation of the sensors was performed by cyclic voltammetry (CV) in 0.5 mol L^{-1} KCl solution containing 10 mmol L−1 [Fe(CN)6]3-/4−. The EIS measurements were carried out by applying a fixed potential of + 1.0 V in 0.10 mol L^{-1} KCl solution in the presence and absence of 50 µmol L^{-1} cyclobenzaprine. CV and square-wave voltammetry (SWV) were used for the voltammetric behavioural characteristics, and the determination of cyclobenzaprine.

The analytical curve of cyclobenzaprine was constructed in triplicate by SWV, in which aliquots of cyclobenzaprine were added in the electrochemical cell containing 10 mL of NaCl solution (0.10 mol L^{-1}). The LOD was calculated according to the IUPAC recommendation: LOD = 3 s/m, where s is the relative standard derivation of 10 measurements of the blank solution, and m is the slope of the analytical curve [35].

2.6. Preparation of samples containing cyclobenzaprine

Pharmaceutical samples were prepared using 10 tablets. The tablets were weighted and ground to a homogeneous fine powder in a mortar. A mass corresponding to one tablet was transferred into a 10 mL volumetric flask and completed with 0.10 mol L−1 NaCl solution. An aliquot of 5 µL was added to the electrochemical cell containing 10 mL of NaCl solution (0.10 mol L^{-1}). The concentration of cyclobenzaprine was determined directly by interpolation from a previously obtained standard analytical curve.

River water samples were collected from Ribeirão Cambé (Paraná, Brazil), and tap water from the city water supply at Universidade Estadual de Londrina (Paraná, Brazil). Both water samples were filtered, and 0.5 mL of the filtrate was transferred to the electrochemical cell containing 9.5 mL of NaCl solution (0.10 mol L^{-1}). These solutions were fortified with the standard solution of cyclobenzaprine of known concentration to evaluate the accuracy of the method of the recovery tests.

3. Results and discussion

3.1. Morphological and electrochemical characterisation of the CB/GCE and CB-BOT/GCE

The surface morphology of CB/GCE and CB-BOT/GCE was investigated by SEM. Fig. 1 shows the SEM images for CB (Fig. 1A) and CB-BOT (Fig. 1B), which in both cases, it is possible to observe that the CB is covering all the electrode surface and has a relatively high porosity from the morphology of CB. The SEM images do not reveal any significant morphological differences on the electrode surface when modified only with CB, or when modified simultaneously with CB and BOT. Note, that the use of BOT is very important because it provides a better adherence of CB upon the electrode surface, which improves the electrochemical response for the sensor, in terms of stability and repeatability, which was not observed for GCE modified just with CB (see Section 3.2.1 later). For CB/GCE, losses of CB into the electrolyte solution occurred during electrochemical measurements containing the redox pair [Fe(CN)6]3-/4− or cyclobenzaprine.

The electrochemical characterisation and benchmarking of bare GCE, BOT/GCE, and CB-BOT/GCE electrochemical electrode surface area (Areal) was performed by CV and EIS. The electroactive area (A) of each sensor was calculated from cyclic voltammograms recorded at different scan rates ranging from 5 to 100 mV s^{-1} in 0.5 mol L−1 KCl solution containing 10 mmol L−1 [Fe(CN)6]3-/4−. The graph of peak current (Ip) vs. square-root of scan rate (v1/2) was plotted, and its linear relationship between Ip and v1/2 is predicted by the Randles–Ševčík equation [36]. By this equation the electroactive areas were estimated as 0.0136, 0.0138, 0.0219, and 0.0255 cm^2, respectively, for GCE, BOT/GCE, CB/GCE and CB-BOT/GCE. It was possible to observe a significant increase (87%) between the GCE and CB-BOT/GCE, which is due to the nature of CB, a nanomaterial with an average particle size of 10 nm. Although BOT is macromolecular, it aids to improve the stability of the sensor. When the RSD for the cyclobenzaprine responses using electrodes without BOT (CB/GCE) and with BOT (CB-BOT/GCE) are compared, CB/GCE does not have sufficient stability (e.g.: RSD 65%, N = 5), and is accompanied by the loss of CB from the electrode surface into the supporting electrolyte solution, and this may have also been responsible for CB/GCE having a lower electroactive area compared to CB-BOT/GCE.

EIS measurements were applied to evaluate the electrochemical response of different sensor surfaces and were carried out by applying a fixed potential of + 1.0 V in 0.10 mol $^{-1}$1 KCl solution in the presence of 50 µmol L^{-1} cyclobenzaprine. BODE spectra of the electrodes were analysed (Fig. S2), which carries important system information to propose an equivalent circuit model [37,38], see supplementary material. Fig. 2 presents the Nyquist spectra of the three electrodes developed, as well as the proposed equivalent circuit models that were used in the mathematical adjustments to obtain the interfacial parameters. For the present work, two models were used: (i) adsorption model (Fig. 2D) [39] for the GCE and BOT/GCE platforms; and (ii) a model with two in-series RC (Fig. 2E) for the CB-BOT/GCE platform. For better mathematical adjustment, the capacitive elements were replaced by constant phase elements (CPE). CPE here was interpreted as a non-ideal capacitor accompanied by the factor [37,40]. Alpha can take values in the range of 0 to 1, with a value of 1 being the ideal capacitor. The models presented a chi-square of < 10−3. Thus, the model in Fig. 2D presented three resistive elements (i) resistance for the solution (0.10 mol L^{-1} KCl solution containing 50 µmol L^{-1} cyclobenzaprine) (RΩ), (ii) charge transfer resistance R1 related to the oxygenated groups (quinone or aldehyde groups) present on the surface of GCE [41,42], and (iii) adsorption resistance (R2). Also, two

capacitive elements, CPEdl - the capacitance of the double electric layer, and CPE2 - the adsorption capacitance. The model agrees with previous EIS studies based upon a Nafion®-coated GCE [42]. Both of our sensors, the bare GCE and BOT/GCE, were observed not to have resistance associated with the oxidation of cyclobenzaprine. When comparing the values of their spectra with those obtained in the absence of cyclobenzaprine (Fig. S3 and Table S2), an increase in the resistance values was observed for both R1 and R2. The behaviour of increasing Rct values in the presence of the analyte is not consistent with Faradaic processes. This demonstrates that the oxidation redox reaction of the oxygenated groups of the GCE electrode was hindered in the cyclobenzaprine molecule presence. All the parameter values obtained from the fitting of the electrochemical impedance spectra are presented in Table 1. The electrode containing CB (Fig. 2E) showed faradaic resistance (R2) for the oxidation of cyclobenzaprine, and the circuit model presented a resistance of the solution (0.1 mol L^{-1} KCl) (RΩ), and a charge transfer resistance related to the CB/GCE interface (R1), in addition to the double layer capacitance and electrochemical capacitance (CPE2) [41]. By comparison, the CB-BOT/GCE platform showed the lowest R2 value related to the others electrodes, probably due to a more homogeneous distribution of CB over the electrode surface provided by the use of BOT, corroborating with the data obtained by cyclic voltammetry. Also, the values of αdl and α2 point to a uniform distribution of CB species on the surface of the GCE electrode when using BOT.

3.2. Improving the performance of CB-BOT/GCE as a working electrode

3.2.1. Electrochemical behaviour of cyclobenzaprine on the CB-BOT/GCE

An electrochemical study via cyclic voltammetry was performed with the purpose of evaluating the response of cyclobenzaprine on the different surfaces: GCE, BOT/GCE, CB/GCE, and CB-BOT/GCE (Fig. 3). As can be seen, for all sensors, cyclobenzaprine exhibited an electrochemical oxidation peak with a potential of + 1.0 V, while BOT/GCE did not show any oxidation process for cyclobenzaprine, as studied in this voltammetric window. A reduction process/peak was not observed indicating that electrochemical detection of cyclobenzaprine reaction on the electrodes studied are irreversible.

The lowest current intensity for cyclobenzaprine obtained using BOT/GCE is shown in Fig. 3. BOT can block the active sites on the surface of the BOT/GCE and this is accompanied by a decrease in the electron transfer on the surface of the electrode. For CB-BOT/GCE, cyclobenzaprine presented a higher current intensity and lower potential compared to the other electrodes examined. CB is a nanomaterial and provides an electrocatalytic effect. On using the CB/GCE, CB is lost from the electrode into the supporting electrolyte was observed. This is an undesirable effect in using this sensor type, and confirms the lower current intensity observed. It is important to note that the amount of CB in the composition of the film used is 2.0 mg mL^{-1}, this is 100% more concentrated than the values reported in the literature [24]. Thus, this reinforces that BOT is very important for maintaining the stability of the fabricated CB-BOT/GCE and increases the possibility of using high amounts of CB.

To prove the effect of BOT, a repeatability study of the current intensity obtained from BOT/GCE and CB-BOT/GCE was performed in 0.1 KCl mol L^{-1} solution, in the presence of 20 µmol L^{-1} cyclobenzaprine, using CV (50 mV s^{-1}). After 30 measurements, the loss of CB that occurred from the CB/GCE surface

into the KCl solution indicated a RSD of 11.5% (N = 10). For CB-BOT/GCE, no loss of CB occurred and a RSD of 5.9% (N = 30) was recorded. For this reason, the sensor CB-BOT/GCE was chosen for further experiments.

The CB concentration in DMF was evaluated for the surface modified GCE. The values were 1.0, 2.0, and 3.0 mg mL^{-1}, and the voltammogram results are presented in Fig. S4. In this study, the volume of BOT (concentration of 0.78 mg mL−1) used for all of the experiments was 3 µL. For better repeatability, the amount of CB selected was 2.0 mg mL^{-1} (1.64%) for the modification of GCE. Next, the proportion of CB to BOT was examined. In this case, aliquots of CB dispersion (2.0 mg mL^{-1}; in DMF) and BOT solution (0.78 mg mL^{-1} in water) were adjusted in the proportions: 2:4, 3:3, and 4:2 (CB:BOT, v/v). The proportion of CB:BOT of 3:3 (v/v) presented a higher current intensity, and was therefore chosen for the electrochemical determination of cyclobenzaprine.

3.2.2. Study of supporting electrolyte and scan rate

The supporting electrolyte was investigated using 1.0 mol L^{-1} solutions of KCl, NaCl, BaCl2, K2SO4, and Na2SO4 in the presence of 50 µmol L−1 cyclobenzaprine, using CV at 50 mV s^{-1} (Fig. S5). Due a higher current intensity, NaCl solution was selected as the supporting electrolyte. The effect of concentration of NaCl solution within the range of 0.050 to 0.20 mol L^{-1} was examined, and the best voltammetric response, in terms of higher current intensity and better definition of peak, was observed with 0.1 mol L^{-1} NaCl, and was subsequently selected for the developed method.

The effect of the scan rate on the oxidation potential by cyclobenzaprine at 50 µmol L^{-1} concentrations was investigated withing the range of 5 to 250 mV s−1 using the CB-BOT/GCE in 0.1 mol L−1 NaCl solution. Fig. 4(A) shows the cyclic voltammograms. A shift of the oxidation peak towards a positive direction was observed with an increase in the scan rate, which is one of the characteristic features of an electrochemically irreversible process [43]. The plot of the peak current intensity (Iap) vs. the scan rate (υ) (Fig. 4(B)) shows a linear response indicating that the redox reaction on the surface of the CB-BOT/GCE is controlled by the adsorption of cyclobenzaprine on the electrode surface [43]. The plot of the logarithm of current vs. the logarithm of scan rate (Fig. 4(C)) exhibits a slope of 0.946 which confirming that the redox reaction is controlled by the adsorption of cyclobenzaprine on the electrode surface [43].

The number of electrons transferred (n) in the oxidation of cyclobenzaprine on CB-BOT/GCE was estimated from the equation: Eap– Eap/2 = 47.7 (mV)/αn [44], where Eap is the peak potential, Eap/2 is the half current potential, and α is the transference coefficient (0.5 commonly employed for irreversible systems) [43]. For this purpose, a cyclic voltammogram at 50 mV s−1 was used. The values for Eap and Eap/2 obtained were 0.98 and 0.91 V, respectively. Consequently, the value for n was estimated as 1 electron. The possible mechanism for cyclobenzaprine electrochemical oxidation occurs at the tertiary amine moiety [45].

3.3. Development of the voltammetric method for the determination of cyclobenzaprine

SWV and differential pulse voltammetry (DPV) were employed to demonstrate the electroanalytical technique for voltammetric quantification of cyclobenzaprine. The SWV and DPV parameters were optimized in 0.10 mol L−1 NaCl solution containing 50 µmol L−1 cyclobenzaprine. The SWV ranges investigated were: 10–70 mV, for the pulse amplitude (a); 1–8 mV, for the scan increment (ΔES); 1–30 Hz, for the square wave frequency (f). The selected values were: a = 50 mV; ΔES = 7 mV; f = 20 Hz. For DPV, the investigated ranges were: 50–200 mV, for the pulse amplitude; 5–20 mV s−1, for the scan rate (υ); 10–25 ms, for the modulation time (t), and the selected values were: a = 150 mV; υ = 15 mV s−1; t = 20 ms.

After optimizing the parameters of SWV and DPV, the analytical curves for cyclobenzaprine were obtained using both techniques. Table 2 summarises the analytical parameters obtained, and the SWV technique was found to provide better linear adjustment (r2 = 0.998), higher sensitivity (slope), and a lower LOD value for the voltammetric determination of cyclobenzaprine. Consequently, SWV was selected as the voltammetric technique for the quantification of cyclobenzaprine. The SWV analytical curve was obtained from the peak current, recorded in the voltammograms presented in Fig. 5(A), as a function of cyclobenzaprine concentration. A linear relationship was observed between the peak current and the concentration of cyclobenzaprine within the range 2.0 to 20.6 µmol L^{-1} (Fig. 5(A)), and its respective analytical curve (Fig. 5(B)), with the following linear regression equation I (µA) = −1.5 + 0.805 ([cyclobenzaprine] (µmol L^{-1})) (r2 = 0.998), and the calculated LOD was 0.627 µmol L^{-1} [35].

The stability of the CB-BOT film on the surface of GCE and the intra- and inter-day repeatability by the voltammetric method were investigated. First, the reproducibility of the CB-BOT film was examined using three different dispersions of CB in DMF, and these were used to modify the GCE surface. All measurements were performed by SWV in 0.10 mol L^{-1} NaCl solution containing 20 µmol L^{-1} cyclobenzaprine. The RSD obtained for the three different dispersions was 3.72% and indicates great reproducibility for the modified GCE. Under the same experimental conditions, the stability of the CB-BOT/GCE was investigated. After 60 measurements, the current intensity of cyclobenzaprine was lower than 5.0%. Judging from the data obtained, it is possible to confirm that the sensor displayed excellent reproducibility and stability when the electrode was modified by the film provided by BOT, which was due to the greater adherence of CB on the GCE surface.

The intra-day repeatability was performed in 0.10 mol L−1 NaCl solution in the presence of 20 µmol L^{-1} cyclobenzaprine, and the RSD obtained was 3.31% (N = 10). The inter-day repeatability was performed over five different days under the same experimental conditions resulting in a RSD of 4.65% (N = 5). The results obtained demonstrated good measurement accuracy for the developed method using CB-BOT/GCE combined with the SWV technique.

The analytical parameters for the voltammetric determination of cyclobenzaprine using CB-BOT/GCE were compared with different analytical methods previously described in the literature (Table 3). As seen in Table 3, the electrochemical techniques were more sensitive than the chromatographic and spectroscopic methods for the determination of cyclobenzaprine. In addition, the work presented herein has demonstrated the highest sensitivity of the methods reported. Also, our work presented a

lower LOD when compared with the spectrophotometry and potentiometry techniques. On the other hand, when compared against the chromatographic techniques (HPLC, LC-ESI), the SWV obtained values presented a higher LOD (0.36×10^{-3} [4] vs. 0.627 µmol L^{-1} – our work). However, these techniques require the use of high-purity organic solvents, expensive apparatuses and equipment, and are of time-consuming analysis, as assessed against the simplicity, rapidity, and low-cost instrumentation required by voltammetry methods. 3.4. Analytical application

In order to prove the selectivity of the CB-BOT/GCE, a study was performed on interferents typically found in pharmaceutical formulations, rat blood serum, river and tap water, such as, monocrystalline cellulose, lactose monohydrate, titanium dioxide, ascorbic acid, glucose, uric acid, and inorganic ions as Na^{+}, Zn^{2+}, Ca^{2+}, and NO_3^{-} in the proportions of 1:1 and 1:10 (analyte:interferent). The study was performed in 0.10 mol L^{-1} NaCl solution, in the presence of 20 µmol L^{-1} cyclobenzaprine. The current of cyclobenzaprine obtained in the absence of the interfering substances was compared with those obtained in the presence of each interferent. As can be seen in Table S3, the voltammetric results revealed that there was no significant interference (< 4%) in the determination of cyclobenzaprine.

The CB-BOT/GCE was applied for the voltammetric determination of cyclobenzaprine in pharmaceutical formulations. Table 4 shows the concentration of cyclobenzaprine quantified in the samples using the proposed method, and the comparative method (spectrophotometry) [48]. According to the paired t-test no significant differences were observed between both methods, the calculated t was 0.12, and was smaller than the critical value (4.30), at the 95% confidence level [49,50].

The addition and recovery study were carried out by addition of a known amount of a standard solution of cyclobenzaprine into the sample solution of the pharmaceutical product. Excellent recovery was obtained, ranging from 96.4 to 103%, indicating that there was no important matrix interference in the pharmaceutical samples analysed by the voltammetric method.

Next, the CB-BOT/GCE was applied for the voltammetric determination of cyclobenzaprine in rat blood serum, river and tap water samples. All samples were doped with two different concentrations of cyclobenzaprine: 3.0 and 6.0 µmol L^{-1} for river and tap water; 10 and 15 µmol L^{-1} for rat blood serum. The recovery percentages obtained in triplicate experiments with the CB-BOT/GCE were satisfactory within the range of 94.0 to 103% (Table S4). Besides, the slopes obtained in the standard addition experiments in the rat blood serum sample (representative of a complex matrix) were 0.685 and 0.708 A mol L^{-1}, respectively for the 10 and 15 µmol L^{-1} doping levels. Both results are close to slope obtained in analytical curve (see Section 3.3), indicating no matrix effect in the determination of cyclobenzaprine in complex samples using the CB-BOT/GCE. Thereby, this sensor could be successfully applied for the voltammetric determination of cyclobenzaprine in complex samples such as these.

4. Conclusion

The fungal exopolysaccharide BOT was successfully applied to improve the adherence of CB onto the surface of the GCE and was indispensable for the use of the fabricated sensor. The electrochemical impedance spectroscopy showed that the CB-BOT/GCE increased the electron transfer kinetics when compared with the bare GCE and BOT/GCE. The use of the modified electrode improved the electroanalytical features of the method applied for the detection of cyclobenzaprine, which presented a higher current and a less positive oxidation potential. The voltammetric determination of cyclobenzaprine using the CB-BOT/GCE in pharmaceutical, biological, and environment samples was successful and showed advantages such as simplicity of sample preparation, fast response, and selectivity in complex matrices.

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Table 1Parameters determined from fitting of the electrochemical impedance spectra in Fig. 2. Eapplied = 1.0 V vs. Ag/AgCl. RΩ = 50 Ω cm². The fitting error is ≤ 2%.

Sensor	R_1(kΩ cm^2)	R_2	CPE_{dl}(μF cm^{-2} s$^{\alpha-1}$)	α_{dl}	CPE_2(μF cm^{-2} s$^{\alpha-1}$)	α_2
GCE	0.13	39.4	23.7	0.90	65.7	0.70
BOT/GCE	2.40	87.6	32.7	0.85	1.92	0.61
BOT-CB/GCE	0.68	12.4	66.8	0.84	38.1	0.68

Table 2. Analytical parameters obtained for the quantification of cyclobenzaprine by SWV and DPV in 0.10 mol L^{-1} NaCl solution.

Parameters	Techniques	
	SWV	DPV
Concentration range (μmol L^{-1})	2.0–20.6	16.0–102.3
Slope (A mol^{-1} L)	0.805	0.101
Correlation coefficient (r^2)	0.998	0.989
LOD (μmol L^{-1})	0.627	2.785

Table 3. Comparison of analytical parameters using different techniques for the determination of cyclobenzaprine.

Technique	Concentration range (μmol L^{-1})	LOD (μmol L^{-1})	Sensitivity	Methods characteristics	Reference
HPLC-RIA	$0.45–26.0 \times 10^{-3}$	0.36×10^{-3}	4.36×10^{-6} Area L mol^{-1}	- Use of organic solvents - Time-consuming sample preparation - Expensive equipment	[46]
HPLC-MS-MS	$0.36–181 \times 10^{-3}$	–	0.35×10^{-3} Area L mol^{-1}	- Use of organic solvents - Expensive equipment	[4]
HPLC-UV	$1.82–181 \times 10^{-3}$	–	0.46×10^{-3} Area L mol^{-1}	- Use of organic solvents - Time-consuming analysis - Expensive equipment	[4]
LC-ESI_MS/MS	$0.18–108 \times 10^{-3}$	–	1.50×10^{-6} Area L mol^{-1}	- Expensive equipment - Time-consuming sample preparation	[47]
Spectrophotometry	7.26–36.31	0.18	1.74×10^{-4} Abs L mol^{-1}	- Complex mathematical data treatment	[8]
Spectrophotometry	7.26–58.09	0.73	4.07×10^{-3} Abs L mol^{-1}	- Use of organic solvents - Complex mathematical data treatment	[48]
Potentiometry	10–10,000	6.5	0.05 A L mol^{-1}	- Time-consuming electrode preparation - Greater concentration range - Use of organic solvents	[14]
Voltammetry	2.0–20.6	0.627	0.81 A L mol^{-1}	- Simples and fast sample preparation - Low cost - No need for organic solvents	This work

Table 4. Cyclobenzaprine quantification in pharmaceutical samples using the proposed and the comparative method.

	Cyclobenzaprine [a] (mg/tablet)			
Sample	Label value	Voltammetric	Spectrophotometric	E (%)
A	5.00	4.85	4.88	−0.61
B	10.0	10.4	9.8	6.62
C	10.0	10.5	10.1	3.85

[a] Average of 3 measurements per sample preparation. b. Relative error (%) = 100 × ([voltammetric method − comparative method] / comparative method).

Fig. 1. SEM images of a GCE modified just with CB in DMF (A) and compared with CB with BOT (B).

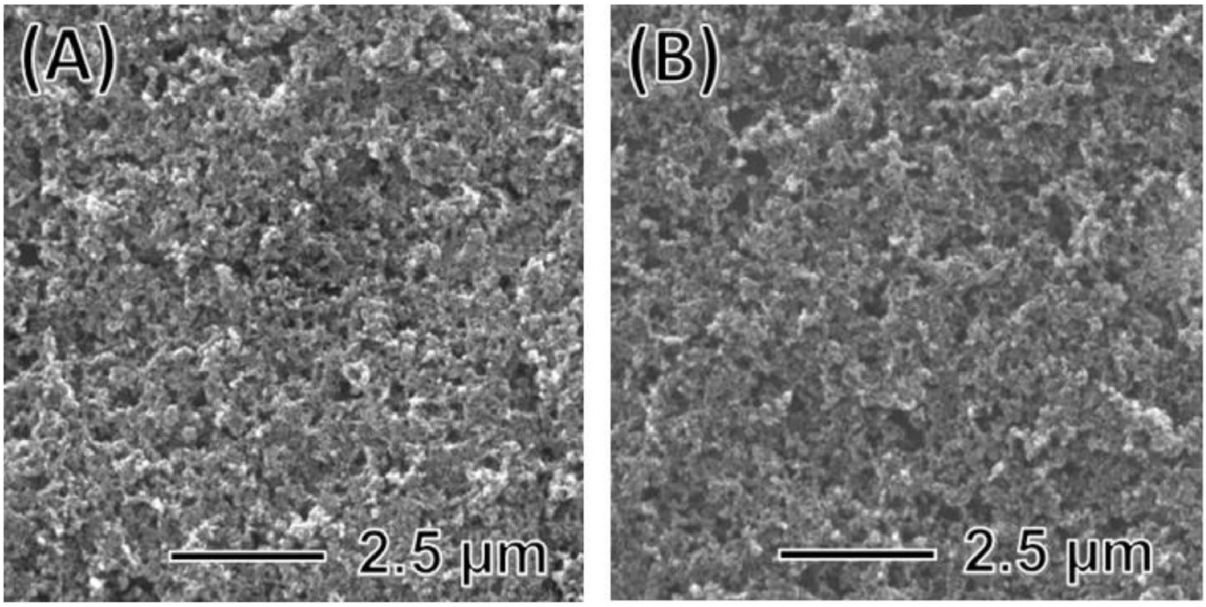

Fig. 2. Nyquist spectra of GCE (A), BOT-GCE (B), and BOT-CB/GCE (C) in the presence of 50 µmol L^{-1} cyclobenzaprine in 0.10 mol L^{-1} KCl solution. The proposed equivalent circuit models are shown for bare GCE and BOT/GCE (D), and for CB-BOT/GCE (E). Insets (A–C): magnification of the respective high frequency regions. Applied potential: + 1.0 V.

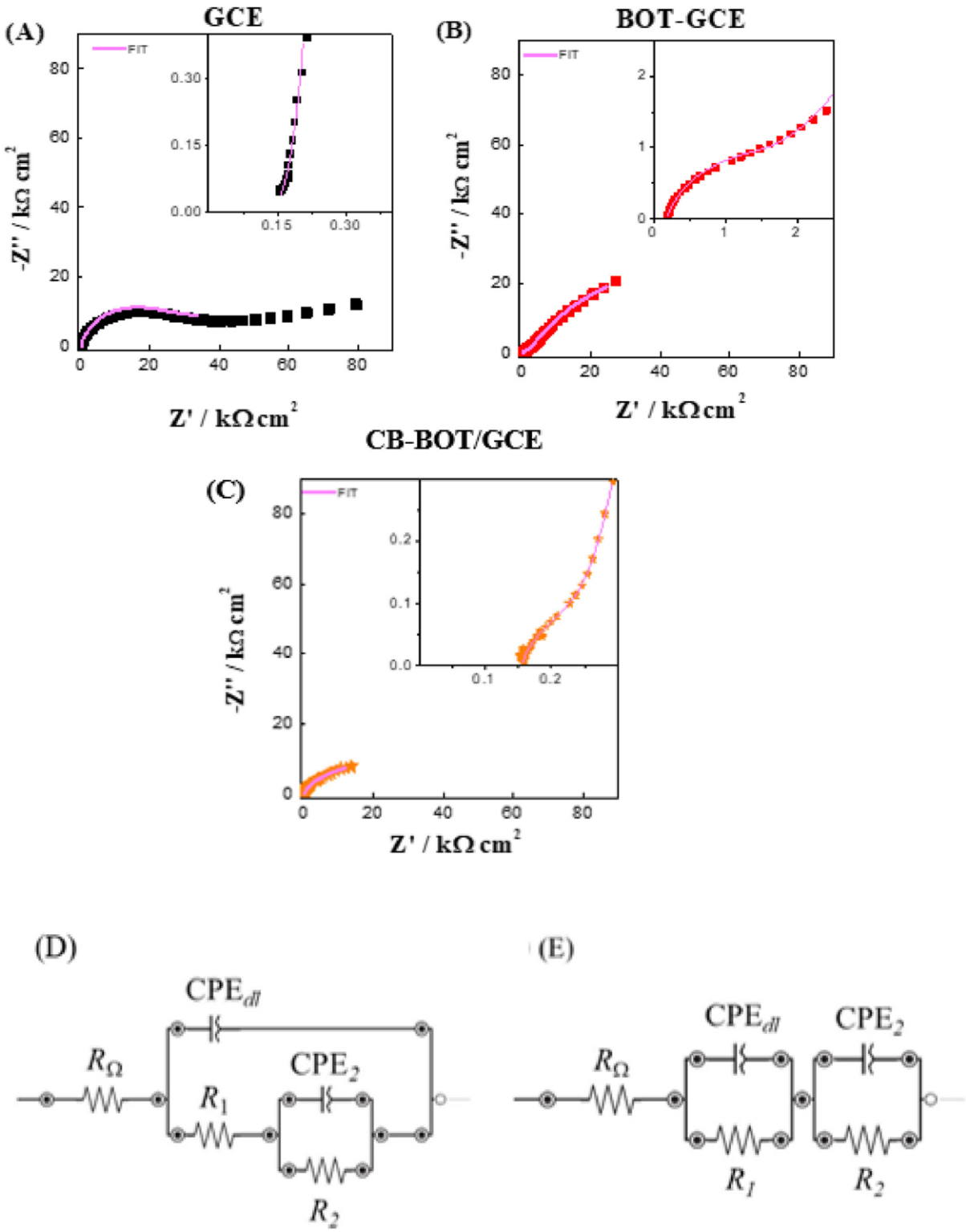

Fig. 3. Cyclic voltammograms (50 mV s−1) in the presence of 50 µmol L^{-1} cyclobenzaprine in 0.10 mol L^{-1} KCl solution, using as working electrode: (—) GCE, (—) BOT/GCE, (—) CB/GCE, and (—) CB-BOT/GCE.

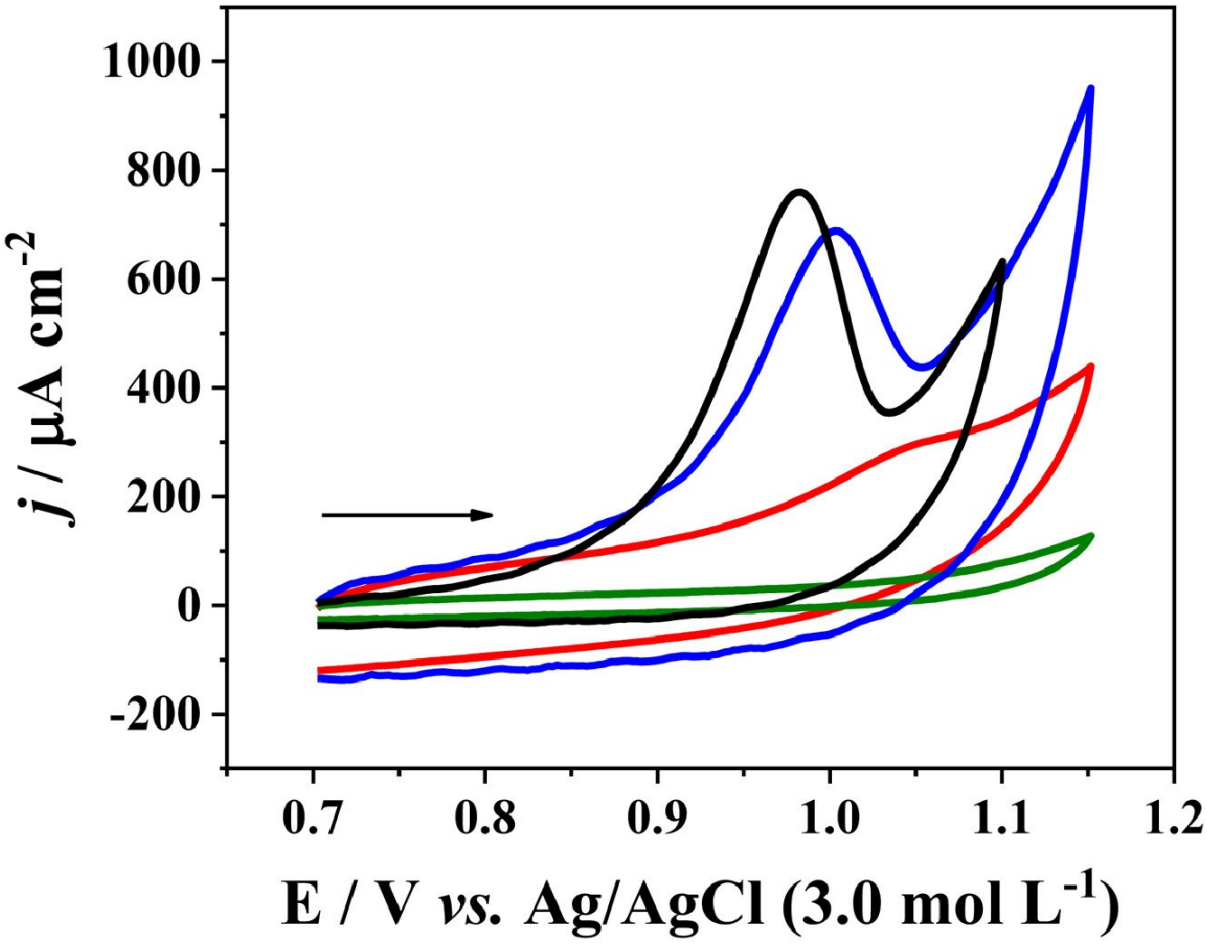

Fig. 4. Cyclic voltammograms obtained with CB-BOT/GCE as the working electrode. (A) Plots of Iap vs. υ (B), and log Iap vs. log υ (C). Scan rate 5–250 mV s^{-1} (a – h), in 0.10 mol L−1 NaCl solution in the presence of 50 µmol L^{-1} cyclobenzaprine.

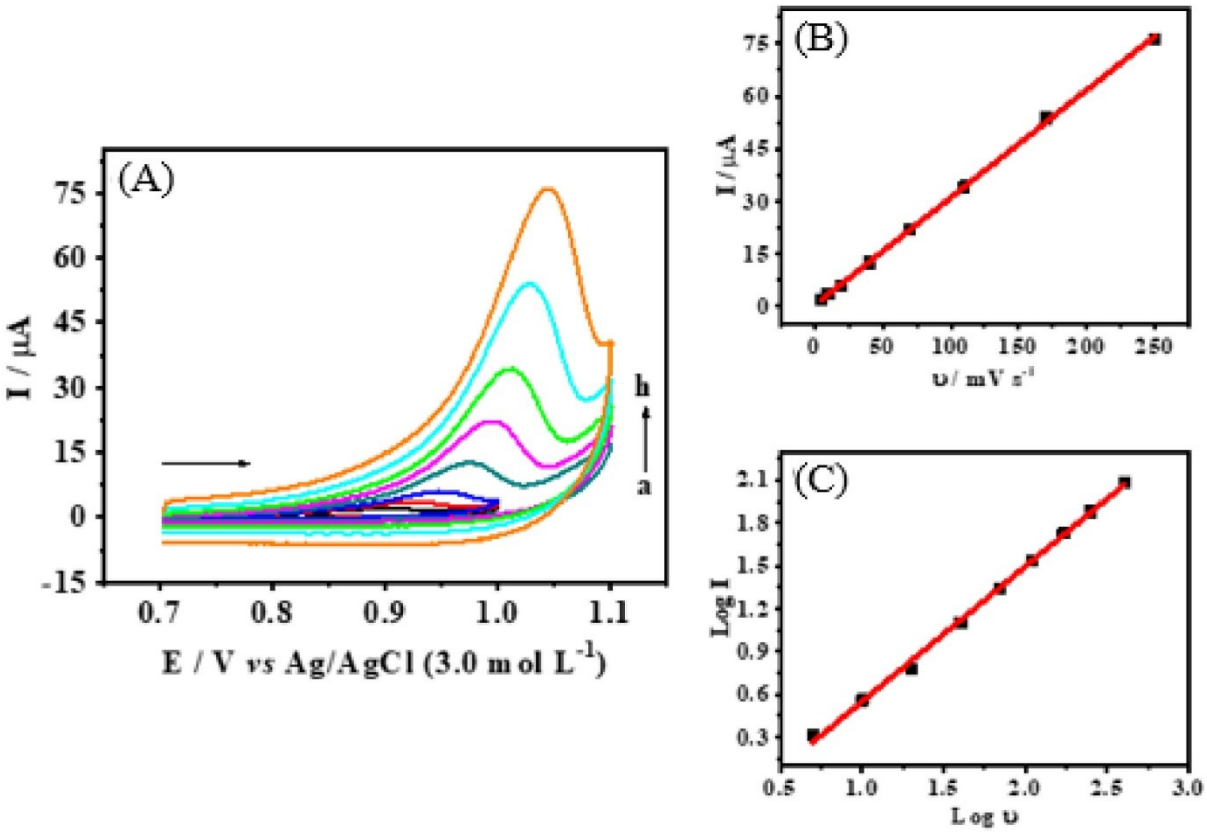

Fig. 5. Square wave voltammograms obtained for cyclobenzaprine within the range 2.0–20.6 µmol L^{-1} (lines 2–9) in 0.10 mol L^{-1} NaCl solution employing CB-BOT/GCE (A). The respective analytical curve relating the concentration of cyclobenzaprine (B).