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VOLTAMMETRIC DETERMINATION OF CHLORAMPHENICOL AT ELECTROCHEMICALLY PRETREATED GLASSY CARBON ELECTRODE

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ABSTRACT. A sensitive square wave voltammetric method for the determination of chloramphenicol (CAP) was developed using electrochemically pretreated glassy carbon electrode (EPGCE). Electrochemical pretreatment of the electrode greatly enhanced the reduction peak current (I_p) of CAP. The electrochemical investigation of CAP was carried out by using cyclic and square wave voltammetry techniques. CAP shows an irreversible reduction peak at -0.646 V *vs.* Ag/AgCl at the EPGCE in 0.05 M CH_3COONa/CH_3COOH buffer of pH 5.3 using the cyclic voltammetric mode. Detailed experiments were carried out to establish the electrochemical property, the optimal pH, electrode pretreatment potential and square wave voltammetric parameters. Following optimization of the instrumental parameters and pH of buffer solutions, the peak current response for the reduction of CAP was observed showing a linear calibration curve in the concentration range of 1.0×10^{-7} - 7.0×10^{-5} M CAP. Over this concentration range, two good linear ranges were obtained between the voltammetric current and CAP concentration. The first was in the linear range 1.0×10^{-7} - 5×10^{-6} M CAP (r = 0.999) and the second in the linear range 5.00×10^{-6} – 7.00×10^{-5} M CAP (r = 0.999). For a series of six determinations of CAP at 1.00×10^{-5} M and 5.00×10^{-7} M levels relative standard deviations of 2.2 % and 3.7 %, respectively were obtained, showing an excellent reproducibility of the EPGCE. When the signal to noise ratio is 3, the detection limit was 6.0×10^{-9} M. The experiment on the possible interfering substances showed that the electrode has excellent selectivity for the detection of CAP. The method was verified by the determination of CAP in eye drops.

KEY WORDS: Chloramphenicol, Electrochemically pretreated glassy carbon electrode, Square wave voltammetry

INTRODUCTION

Electrode pretreatment and surface modifications have been widely used to improve the properties of electrode surface in order to overcome slow kinetics of electrode processes. Various kinds of activation procedures have been developed for pretreating electrodes such as glassy carbon electrode. These include electrochemical activation [1-8], mechanical polishing [9], ultrasonic cleaning [10], electric arc activation [11], laser irradiation [12], and vacuum heating [13]. Among these the electrochemical treatment is one of the most widely used technique that is often carried by potentiostatic polarization at a suitable potential or by potential cycling in a wide range [7]. It is believed that the oxidative pretreatment could produce oxygen containing functional groups such as hydroxyl, carbonyl, carboxyl and quinones on carbon surfaces, which are responsible for the improved performances of carbon electrodes. The surface oxide films have been characterised by different techniques [2-8, 10, 12]. They are widely used to improve the electrochemical responses of biological compounds and construct electrochemical detectors. The relationship between surface structures and the pretreatment processes, however, are still not fully understood.

Chloramphenicol (CAP) {2,2-dichloro-N-[2-hydroxy-1-(hydroxymethyl)-2-(4-nitrophenyl) ethyl] acetamide}, with a structure shown in Figure 1, is a drug obtained first from cultures of the soil bacterium *Streptomyces venezuelae* and was chemically synthesised in 1948 [14, 15]. It

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is a broad spectrum antibiotic with high liposolubility, characteristic that facilitates to cross lipid barriers easily. Among other applications this drug has been used for the treatment of childhood meningitis and typhoid fever. The drug has been used in the veterinary practice for the prevention and treatment of many bacterial infections. However, toxic effects in humans such as aplastic anaemia have been described. Its toxicity is derived from its action on the mitochondria synthesis of proteins that may cause serious secondary effects. These adverse effects have led to restrict its use in both human and veterinary medicine.

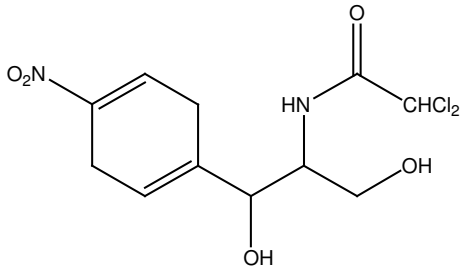

Figure 1. Structure of chloramphenicol (2,2-dichloro-N-[2-hydroxy-1-(hydroxymethyl)-2-(4-nitrophenyl) ethyl] acetamide).

The recommended methods in pharmacopoeias for determining CAP in pharmaceuticals involve UV-spectroscopy and HPLC, but have limited selectivity and ore often subjected to interferences from components of the matrix [14, 15]. Currently used methods for the determination of CAP in animal food samples, milk, meat or tissues and fluids of treated cattle include GC [16-19], HPLC [20-23], and planar chromatography [24]. However, these methods are laborious and require high cost instruments. Electrochemical methods such as polarography [25-28] and voltammetry [29-32] have been used for the determination of CAP by exploiting the partial reduction of the nitro group in an irreversible electrode process. The polarographic method is simple and reproducible. However, the method is limited due to high detection limit and poisonous nature of mercury. The voltammetric methods at solid electrodes that are carried out without electrode surface pre-treatment suffer from electrode poisoning and irreproducibility [29, 30]. Voltammetric determination of CAP at electrochemically activated carbon fibre microelectrodes [31] and at multiwall carbon nanotube-modified electrodes [32] have been reported. However, both of these methods have high detection limits and narrow linear ranges. Since CAP is of great pharmaceutical importance, and due to its toxic effects, sensitive analytical methods for the strict control of CAP in pharmaceutical products are necessary.

In this study a sensitive voltammetric method was developed and is described for the determination of CAP at EPGCE. The EPGCE was achieved in acetate buffer media by both anodic oxidation and potential cycling in a suitable range. The electrochemical behaviour of CAP and its determination in real sample are explained.

EXPERIMENTAL

Apparatus

Voltammetric measurements were performed using a BAS 100B Electrochemical Analyser [Bioanalytical Systems (BAS) USA)] and a one-compartment glass cell vial (BAS MR-1208)

with a three-electrode configuration (BAS Cell Stand C3). The electrodes used were a glassy carbon disk working electrode with a diameter of 3 mm (BAS MF-2012), a platinum wire auxiliary electrode (BAS MW-1032), and an Ag/AgCl (3 M NaCl) reference electrode (BAS MF-2052). The pH of the buffer solution was measured with Hanna instruments digital pH meter with a glass combination electrode. All potentials are reported with respect to Ag/AgCl (3 M NaCl) reference electrode.

Reagents

CAP was obtained from Lesotho Pharmaceutical Corporation, Mafeteng. Acetic acid (LABCHEM, RSA), sodium acetate, sodium dihydrogenphosphate and disodium hydrogen orthophosphate dihydrate (UNILAB SAARCHEM, RSA), sodium hydroxide and hydrochloric acid (Associated Chemical Enterprise, C.C.), boric acid (AnalarR), *o*-nitrophenol (Riedel-de-Haën Germany), 4-nitroacetanilide (Merck-Schuchardt, Germany), 2-chloro-4-nitroaniline (Aldrich Chemical Co. USA) were used as received. Distilled and deionized water was used throughout.

A 0.05 M acetate buffer (pH 5.3) was prepared by dissolving the required amount of sodium acetate in distilled and deionized water and the pH of the solutions was adjusted by addition of drops of acetic acid. Stock solutions of CAP 1 x 10^{-3} M were prepared in distilled and deionized water daily. The working solutions for the voltammetric investigations were prepared by dilution of the stock solution with aqueous buffer solutions. All stock solutions were protected from light by keeping them in the dark and were used within several hours to avoid decomposition.

Electrochemical pretreatment of glassy carbon electrode

The glassy carbon electrode was polished with BAS polishing alumina on a micro-cloth pad and thoroughly rinsed with water. Then it was cleaned in an ultrasonic bath for three minutes. Electrochemical pretreatment of glassy carbon electrode was performed by anodic oxidation at +1.000 V for 60 s in acetate buffer (pH 5.3). The electrode was then cycled between –1.000 V and +1.000 V at a scan rate of 100 mV s^{-1} until a stable voltammogram was obtained. After each electrochemical determination, the solution was stirred for 30 seconds while electrochemically cleaning the surface of the working electrode at +1.000 V, prior to the next measurement.

Procedure

A 10 mL of supporting electrolyte solution was placed in the electrochemical cell and the required volume of the standard CAP solution was added to the cell with a micro-pipette. The same procedure was followed for the sample analysis.

The solution was deaerated with pure nitrogen (99.999 %, Air products SA). Cyclic voltammetric measurements were run from 0.000 to -1.000 V and back. Square wave voltammetric measurements were run from 0.000 to -1.200 V using the Osteryoung square wave voltammetric mode and the net current responses were recorded. The parameters for square wave voltammetric measurements were: the potential step was 14 mV, the square wave amplitude was 50 mV, and the square wave frequency was 130 Hz. All measurements were carried out at room temperature (22 ± 2 ^{o}C).

Sample analysis

The sample analyzed was a CAP eye drops BP 0.5 % (IMRES, Netherlands) which was purchased from a local pharmacy. A 5 mL CAP eye drops solution with a concentration of 5 mg

ml^{-1} was used to prepare 1 x 10^{-4} M stock solution of the sample by dilution with distilled water. An aliquot of this solution (100 µL) was spiked into the electrochemical cell that contained 10 mL of 0.05 M acetate buffer (pH 5.3) and the voltammogram was recorded following the already outlined voltammetric procedure. The standard addition method was then applied, adding successive aliquots of 100 µL of 1 x 10^{-4} M standard CAP solution to the electrochemical cell. Square wave voltammograms were recorded by scanning cathodically from 0.000 to -1.100 V. The net peak current of the reduction wave at -0.650 V was measured. The calibration graph was then constructed by plotting the net peak current against CAP concentration.

RESULTS AND DISCUSSION

Electrochemical pretreatment of glassy carbon electrode

In this study, potentiostatic and cyclic voltammetric methods were used for the electrochemical treatment of glassy carbon electrode. As described in the experimental section, +1.000 V was applied to the freshly polished and cleaned glassy carbon electrode for 60 s in a solution of acetate buffer, and then followed by potential cycles between +1.000 V and -1.000 V in the same solution until a stable background voltammogram was obtained. During these cycles oxygen containing functional groups such as -C=O and -C-OH are formed on the electrode surface and are reduced and oxidised from one form to another [2]. Such functional groups increase the density of active sites at the electrode surface and improve the electron transfer of the reaction. Moreover, the electrode's surface structure becomes very porous after each treatment and hence the electrode surface area increases.

Figure 2 compares the cyclic voltammograms of 1 x 10^{-4} M CAP obtained at a bare glassy carbon electrode and EPGCE, respectively. As it is seen, the peak current of CAP at about -0.645 V obtained at EPGCE is 2.2 times greater than that of the bare glassy carbon electrode. Thus there is a substantial enhancement in the peak currents when the glassy carbon electrode is electrochemically pretreated. Hence the pretreated electrode was employed using square wave voltammetry for the analytical applications of CAP.

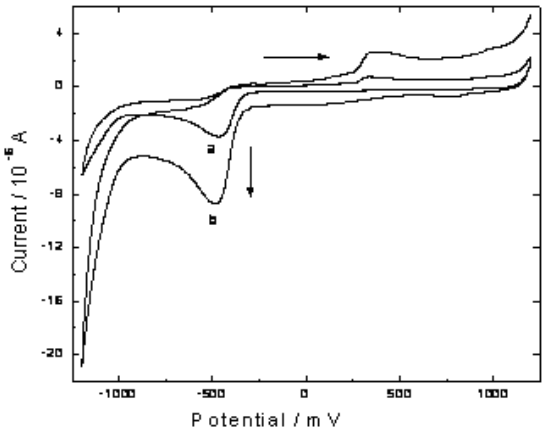

Figure 2. Cyclic voltammograms of: (a) 1 x 10^{-4} M CAP at a bare glassy carbon electrode; (b) 1 x 10^{-4} M CAP at EPGCE; 5 x 10^{-2} M acetate buffer pH 5.3 and scan rate 100 mV s^{-1}.

Electrochemical behaviour of CAP at EPGCE

The electrochemical behaviour of CAP was characterised both by cyclic and square wave voltammetry techniques. Figure 3 shows the cyclic voltammograms of 1 x 10^{-4} M CAP at an EPGCE in pH 5.3 CH_3COONa/CH_3COOH buffer for four continuous cycles. During the first cycle, in the cathodic direction, an irreversible reduction peak A appeared at -0.645 V. On the reverse anodic scan no oxidation peak was observed corresponding to peak A, indicating that the reduction peak is irreversible while an oxidation peak B_1 appeared at 0.310 V. During the second cathodic sweep, a new reduction peak B_2 that is chemically reversible with peak B_1 was observed at -0.024 V. As the number of cycles increased the peak currents of the redox couple (B_1 and B_2) peaks increased while the peak current of peak A decreased. This shows that the product of the irreversible reduction of CAP remained on or near the electrode surface and was oxidised on the anodic sweep. When the potential was scanned while string the solution the peaks at B_1 and B_2 disappeared. Further, when the potential scan was restricted to the potential range 0.200 to -0.500 V, the peaks of B_1 and B_2 disappeared. These observations indicate the electrochemically generated product during the irreversible reduction of CAP (peak A) is responsible for the formation of B_1 and B_2. It is clear from the literature that nitrophenyls undergo irreversible four electron reduction to give N-phenylhydroxylamine [33]. It is also reported that CA undergoes a slow 2-electron reduction of the nitro group which is followed by a fast 2-electron reduction to hydroxyl amine [25]. Hence the observed peaks of CAP in Figure 3 can be described by the following electrochemical reactions.

$$R\text{-}NO_2 + 4e^- + 4H^+ \rightarrow R\text{-}NHOH + H_2O \quad \text{peak A} \tag{1}$$

$$R\text{-}NHOH \leftrightarrow R\text{-}NO + 2H^+ + 2e^- \quad \text{peaks } B_1/B_2 \tag{2}$$

where $R\text{-}NO_2$ represents CAP. In order to further characterise the electrochemical behaviour of CAP, square wave voltammograms were run in the potential range shown in Figure 4, in the anodic direction. The peaks shown in the figure belong to the net current-potential curves of the square wave voltammograms.

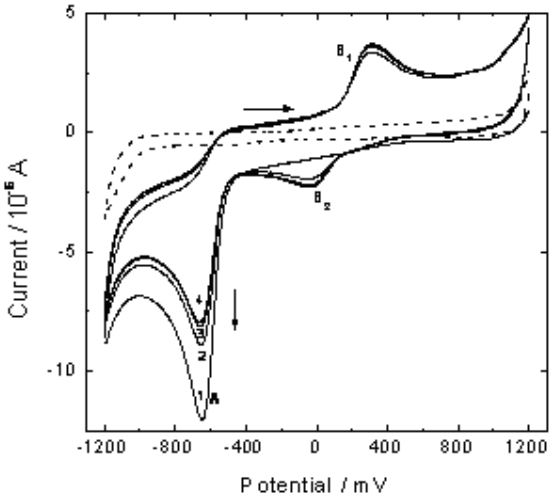

Figure 3. Cyclic voltammograms of 1 x 10^{-4} M CAP for four repetitive cycles in 5 x 10^{-2} M acetate buffer, pH 5.3 (dash line), with scan rate 100 mV s^{-1} at EPGCE.

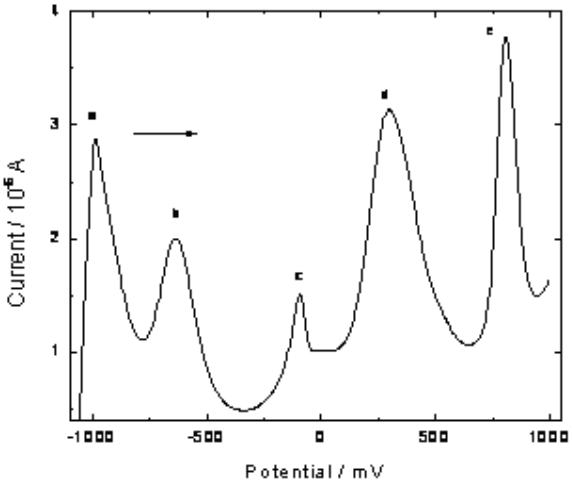

Figure 4. Square wave voltammogram of 1 x 10^{-5} M CAP in 5 x 10^{-2} M acetate buffer, pH 5.3 at EPGCE.

In the Osteryoung square wave voltammetry (OSWV), the perturbation of the potential with time consists of an in-phase combination of a staircase waveform of small and constant step height with periodic square wave pulses. This perturbation consists of pulses alternating in direction, i.e. a succession of forward (reduction, or oxidation) and reverse (oxidation, or reduction) cycles. The faradaic current is sampled at the end of each half cycle. Therefore, the current is sampled twice during each square wave cycle: the forward current and reverse current. The net current is, therefore, the forward current minus the reverse current. It is to be noted that since the forward and the reverse currents have opposite signs, their difference corresponds in absolute to their sum [34, 35].

Five peaks are observed in the range of the potential scan, corresponding to peak **a** at -0.987 V, peak **b** at -0.638 V, peak **c** at -0.093 V, peak **d** at 0.297 V and peak **e** at 0.807 V. The peak at **a**, which is not illustrated in Figure 3 of the cyclic voltammograms, is attributed to the reverse peak for the reduction of the nitro group of CAP to amine group since the nitro group is reduced irreversibly according to the following reaction [25, 36].

$$R\text{-}NO_2 + 6e^- + 6H^+ \rightarrow \quad R\text{-}NH_2 + 2H_2O \qquad (3)$$

The peak at **b** is also attributed to the reverse peak that corresponds to the irreversible reduction of the nitro group to hydroxylamine as shown above by equation (1) and in Figure 3. The peak at **c**, which is not seen in the cyclic voltammograms of Figure 3, is sharp and relatively small; presumably it corresponds to an adsorption peak prior to the oxidation of the hydroxylamine to nitroso group. Peak **d** is assigned to the net peak (i.e. the difference between the forward peak and reverse peak) corresponding to the reversible oxidation and reduction of the hydroxylamine to nitroso group and vice-versa as shown above by equation (2). The last peak at **e** that has not been seen in Figure 3 strongly depends on the initial potential of the scan. It has been observed that the existence of this peak and its intensity is strongly associated with the other peaks. It is believed to be due to the oxidation of the nitroso group to nitro group (assigned to the forward peak) according to the following reaction [33, 37].

$$R\text{-}NO + H_2O \rightarrow R\text{-}NO_2 + 2H^+ + 2e^- \quad (4)$$

The peak at **b** corresponding to -0.638 V exhibited the maximum peak current for CAP in the square wave voltammetric mode after optimising the instrumental parameters and pH of the supporting electrolytes. Moreover, the peak current was found to be proportional to the concentration of CAP; hence, this peak was systematically studied by square wave voltammetry for the detection of CAP.

Influence of buffer and pH of supporting electrolyte

A series of buffer solutions as supporting electrolytes were tested for their suitability in the determination of CAP. These include acidic buffer, KCl/HCl; acetate buffer, CH_3COOH/CH_3COONa; phosphate buffer, KH_2PO_4/Na_2HPO_4; and borate buffer, $Na_2B_4O_7.10H_2O/NaOH$. The peak height and shape of the voltammograms were considered for the choice of the supporting electrolytes. The optimum buffer solution chosen for subsequent studies was acetate buffer.

The influence of pH on the peak current of CAP was investigated over the range of pH 0.9 – 11.0. Figure 5 compares three linear scan voltammograms of CAP solution obtained at pH 0.9, 5.3 and 11.0, respectively. As it can be seen, there is a variation in current and potential with pH. When the pH of the supporting electrolyte is increased, the peak of the voltammograms is shifted to a more negative potential. The peak current obtained in a buffer of pH 11.0 is much less than that obtained for the buffer solutions of pH 5.3 and 0.9. Figure 6 shows the dependence of the peak current on pH for the cyclic voltammetry measurements. The peak current is low at high pH ranges and starts increasing as the pH decreases and reaches a maximum value at around pH 5.3. Then it decreases slightly and levels of at low pH. The high current values in acidic buffer solutions are expected since the reduction of the nitro group of CAP to hydroxylamine involves H^+ ions as shown by equation (1).

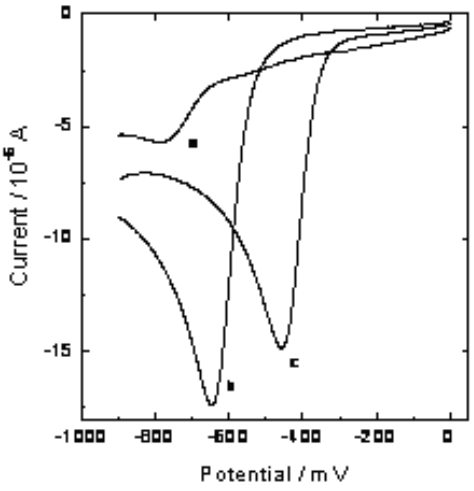

Figure 5. Linear scan voltammograms of 2 x 10^{-4} M CAP in: (a) 5 x 10^{-2} M $Na_2B_4O_7.10H_2O/NaOH$ buffer (pH 11.0); (b) 5 x 10^{-2} M CH_3COONa/CH_3COOH buffer (pH 5.3); (c) 5 x 10^{-2} M KCl/HCl buffer (pH 0.9), with scan rate 100 mV s^{-1} at EPGCE.

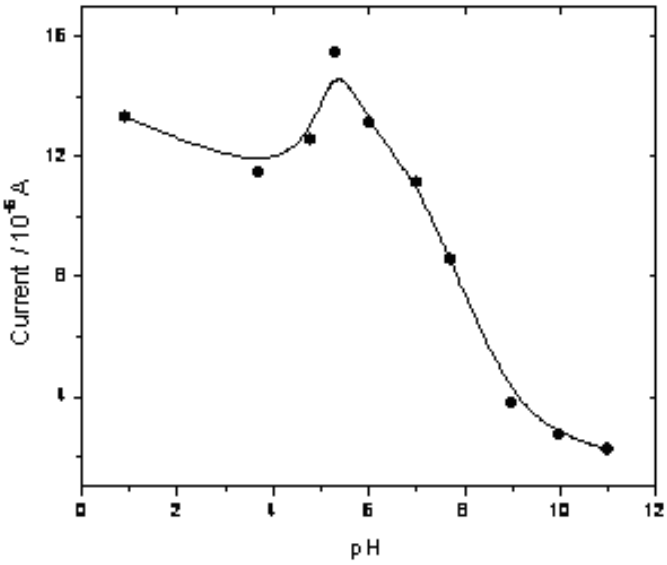

Figure 6. Plot of peak current as a function of pH for the cyclic voltammetry responses of 2 x 10^{-4} M CAP.

The shift in the cyclic voltammetry peak potential as a function of pH was studied and linear dependence was observed (figure not shown). Similar peak potential shift and linear dependence on pH was obtained when the pH was varied using the square wave voltammetry mode. A linear range which is described by the following equation:

$$E_P/V = 0.056pH - 0.686; \qquad r = 0.996 \tag{5}$$

The dependence of the peak potential on the pH has slope of 56 mV per unit pH. This implies that the ratio of the number of protons to the electrons is 1:1 for the step in which the electrode process is reversible [38] which is in accordance to equation (1). Electrode processes involving a weak acid or weak base have a potential-pH variations which show a change in slope at pH = pK_a. In the potential-pH array there was only one linear plot indicating that the pK_a of CAP is out of this range. It is reported in the literature that CAP has pK_a value of 11.03 [39].

Voltammetric parameters

The instrumental parameters in square wave voltammetry are interrelated and have a combined influence on the peak current [34]. Hence, in order to establish the optimum conditions in the determination of CAP, the influence of instrumental parameters on the current response was studied.

The influence of the pulse amplitude (ΔE) on the peak current was studied in the range 25 to 65 mV. The peak current increased sharply up to 50 mV then reached a steady state value. The ΔE was then set at 50 mV for the subsequent measurements. The effect of the potential step (ΔE_s) on the peak current was also investigated in the range 4 to 18 mV. The plot of the peak current as a function of ΔE_s increased sharply at the beginning and continued increasing gently. A potential step of 14 mV was chosen as the optimum value for the analysis. The square wave

frequency (*f*) was varied from 5 to 150 Hz. At lower frequencies the current response was very low. At high frequencies the peak current increased almost linearly with increase in *f*. However the shape of the voltammograms became broader as *f* increased. A square wave frequency of 130 Hz was chosen to be the optimum value. The effect of the initial sweep potential on the peak current was also examined in the potential range 1200 to -250 mV. The peak current did not show significant change within the range and an initial sweep potential of 0 mV was used for all subsequent measurements. Finally the instrumental parameters selected were: ΔE = 50 mV, ΔE_s = 14 mV, f = 130 Hz and initial sweep potential = 0 mV. Figure 7 illustrates the square wave voltammogram of 2 x 10^{-4} M standard solution of CAP using the optimised instrumental parameters.

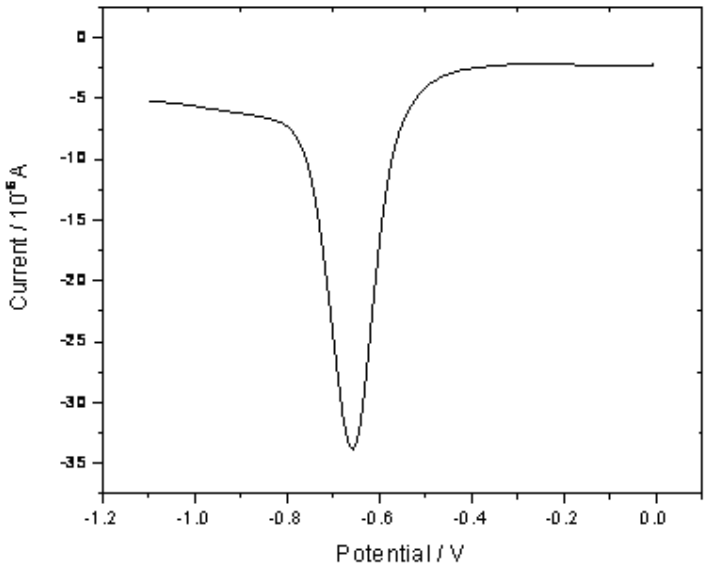

Figure 7. Square wave voltammogram of 2 x 10^{-4} M CAP at EPGCE in 0.05 M CH_3COONa/CH_3COOH buffer (pH 5.3), with step potential of 14 mV, square wave amplitude of 50 mV and square wave frequency of 130 Hz.

Linear range and detection limit

The inherent sensitivity of the method is illustrated by the square wave voltammograms at different concentrations of CAP (figures not shown). The net peak current was found to be directly proportional to the bulk concentration of CAP in the concentration range of 1.0 x 10^{-7} - 7.0 x 10^{-5} M CAP. Over this concentration range, two good linear ranges were obtained between the voltammetric current and CAP concentration under the optimum experimental conditions. The response was found to be linear in the concentration ranges 1.0 x 10^{-7} - 5 x 10^{-6} M CAP (r = 0.999) and the second in the linear range 5.00 x 10^{-6} – 7.00 x 10^{-5} M CAP (r = 0.999). For the regression plot of the peak current versus CAP concentration, the slope = 1165 μA μM^{-1}, the y intercept = 3.95 μA for the lower concentration range, and slope = 204 μA μM^{-1}, the y intercept = 10.16 μA for the higher concentration range. For a series of six determinations of CAP at 1.00 x 10^{-5} M and 5.00 x 10^{-7} M levels relative standard deviations of 2.2 % and 3.7 %, respectively were obtained, showing an excellent reproducibility of the EPGCE. When the signal to noise ratio is 3, the detection limit was 6.0 x 10^{-9} M. When the data were collected in a given

experiment for concentrations 1.0 x 10^{-7} - 7.0 x 10^{-5} M, a large value of the intercept was observed for the higher concentration range (5.00 x 10^{-6} – 7.00 x 10^{-5} M) as observed above. Whereas the intercept calculated from data obtained when the experiment was run only for concentrations 5.00 x 10^{-6} – 7.00 x 10^{-5} M was very low and comparable to the intercept of the low concentration range (1.0 x 10^{-7} - 5 x 10^{-6} M).

The analytical results obtained in this work are compared with the results of CAP determination using chemiluminescence method [40]. The chemiluminescence method gave analytical calibration curve within the range 5 x 10^{-5} and 1 x 10^{-3} M with a limit of detection of 1 x 10^{-5} M. This detection limit is very high as compared to the value obtained in the present study and as a result the chemiluminescence method cannot be utilized for the detection of CAP at low concentrations.

Interferences

The effect of the preservative associated with CAP in its pure form and its formulations were tested using the developed method. This method does not suffer any interference from commonly associated preservative agents in the preparation of eye drops such as phenylmercuric nitrate. Other substances tested as potential interferents were other antibiotics such as neomicine, oxytetracycline, tetracycline, and chlorotetracycline; a sulfa-drug such as sulfamethazine; thyreostatics such as methylthiouracil, thiouracil and propylthiouracil; and a diuretic compound such as furosemide. The presence of these compounds up to a concentration of 1 x 10^{-4} M did not affect the response of CAP and none of them showed a characteristic square wave response under the experimental conditions used for the determination of CAP. The effects of nitro group containing organic compounds on the peak current of CAP were tested using the developed method. These include 2-chloro-4-nitroaniline, 4-nitroacetanilide, 2-nitrobenzoic acid, 4-nitroaniline, 2-nitroaniline, 2-nitrophenol, and niclosamide. The presences of these compounds up to a concentration level of 1 x 10^{-6} M enhanced the peak current of CAP and interfere in its determination. However, these compounds do not exist in CAP formulations.

Analytical application

The proposed square wave voltammetric method was used in the determination of CAP in CAP eye drops. The analysis of CAP eye drops was carried out using the standard addition method in the concentration range that fell within the linear range of CAP concentrations. A linear standard addition curve was obtained, with gradient of 760283.77 μA mM^{-1}, y-intercept of 3.63 μA, and correlation coefficient, $r^2 = 0.999$. The content of CAP in the eye drop was obtained as a mean value of 5.2 mg mL^{-1}. This is in very good agreement with the declared value of 5.0 mg mL^{-1}.

CONCLUSIONS

The method described in this work has shown that CPA can be determined by square wave voltammetry using electrochemically pretreated glassy carbon electrode with electrochemical stability in pH 5.3 acetate buffer solution. The electrochemical pretreatment, the buffer system and the optimised instrumental parameters were found to greatly influence the sensitivity of the voltammetric method. This method was successfully applied for the determination of CAP in pharmaceutical formulations in the form of eye drops. The method is simple, relatively faster, has a wider linear range, and has a better detection limit in comparison with other methods used previously for the study of CAP, especially other voltammetric methods.

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