

A flow injection method for the analysis of tetracycline antibiotics in pharmaceutical formulations using electrochemical detection at anodized boron-doped diamond thin film electrode

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Abstract

A method using flow injection (FI) with amperometric detection at anodized boron-doped diamond (BDD) thin films has been developed and applied for the determination of tetracycline antibiotics (tetracycline, chlortetracycline, oxytetracycline and doxycycline). The electrochemical oxidation of the tetracycline antibiotics was studied at various carbon electrodes including glassy carbon (GC), as-deposited BDD and anodized BDD electrodes using cyclic voltammetry. The anodized BDD electrode exhibited well-defined irreversible cyclic voltammograms for the oxidation of tetracycline antibiotics with the highest current signals compared to the as-deposited BDD and glassy carbon electrodes. Low detection limit of 10 nM (signal-to-noise ratio = 3) was achieved for each drug when using flow injection analysis with amperometric detection at anodized BDD electrodes. Linear calibrations were obtained from 0.1 to 50 mM for tetracycline and 0.5–50 mM for chlortetracycline, oxytetracycline and doxycycline. The proposed method has been successfully applied to determine the tetracycline antibiotics in some drug formulations. The results obtained in percent found (99.50–103.01%) were comparable to dose labeled.

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1. Introduction

Tetracyclines are broad spectrum antibiotics for their high activity against nearly all gram-positive and gram-negative bacteria. Tetracycline, chlortetracycline, oxytetracycline and doxycycline are commonly used in food protection animals (including honeybee), because of their broad spectrum activity and low production cost. However, tetracycline residues in milk or meat are also toxic and can cause allergic reactions in some hypersensitive individuals in human. Owing to their extensive use in infectious diseases therapy, there have been several analytical methods available for these compounds in pharmaceutical preparations, biological samples and milk samples. Official methods such as microbiological-based techniques can also provide both qualitative and quantitative analysis for tetracyclines [1–3]. These methods have char-

acteristic disadvantages: microbiological tests are not only time consuming, laborious and expensive but also poor in term of sensitivity and selectivity. There are also some batch procedures which employ spectrophotometry [4,5], chemiluminescence [6–9], spectrofluorimetry [10], and electrochemical method [11].

Flow injection (FI) is a well-known tool that offers improvement in most batch methods, especially the high sample throughput. For the tetracyclines, there are some FI methods available with all types of detection such as spectrophotometry [12,13], chemiluminescence [14,15] and electrochemical method [16–18]. The spectrophotometric FI method detections [12,13] for tetracyclines are based on the formation of a color product by their reaction with 4-aminophenazone and hexacyanoferrate(III). However, this method has a limited concentration range. Under the optimized conditions, tetracyclines were determined in the ranges of 1–20 and 20–250 mg L⁻¹. The lowest limit of detection is 0.2 mg L⁻¹ for doxycycline [12]. For the chemiluminescence method [14], a highly toxic and expensive agent,

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N-bromosuccinimide is used. The reported working range are 0.05–3.00 mg L⁻¹ for tetracycline, 0.50–5.00 mg L⁻¹ for oxytetracycline and 0.50–7.00 mg L⁻¹ for doxycycline and chlortetracycline.

Electrochemical techniques such as amperometric and potentiometric detection are good alternative methods that are widely used in pharmaceutical applications. The techniques are usually easy to operate, fast and inexpensive. The sensitivity of electrochemical methods is often greater than the spectrophotometric method. For example, the detection limits using HPLC amperometric detection varied from 0.1 to 1 mg L⁻¹ for tetracycline family [19].

Procedures in electroanalysis strongly depend on working electrode materials, so the searching and development of new electrode material is necessary. Boron-doped diamond (BDD) thin film electrodes have been emerged as new electrode material in the many fields in electrochemistry, especially in electroanalysis applications [20–29]. The most importance electrochemical properties are: (i) the wide working potential window in aqueous and non-aqueous media; (ii) a stable and low background current, leading to improved signal-to-background and signal-to-noise ratios; (iii) slight adsorption of polar molecules and long term stability of the response, leading to improved resistance to electrode deactivation and fouling. Recently, a diamond electrode has been chemically pretreated by electrochemical oxidation [30–32]. The anodized diamond electrodes retain the excellent properties of the as-deposited diamond electrode even though, the electrode surface of anodized diamond electrode is applied by a high anodic potential. Anodized diamond electrodes are suitable for determination of various analytes such as chlorophenol [30], dopamine and uric acid in the presence of ascorbic acid [31] and homocysteine [32]. These anodized electrodes have exhibited some attractive properties such as excellent stability and high reproducibility. In this present work, the anodized diamond electrode was used for the electrochemical determination of tetracyclines using cyclic voltammetry. Glassy carbon (GC) and as-deposited diamond electrodes were also used for comparison. The anodized diamond electrode was exploited as working electrode for amperometric determination of antibiotic drugs, using flow injection system.

2. Experimental

2.1. Chemicals and reagents

All reagents were analytical reagent grade. All solutions were prepared in ultrapure water. Standards (hydrochloric forms) of tetracycline, chlortetracycline, oxytetracycline and doxycycline were supplied by Sigma Chemical (USA). Potassium dihydrogen phosphate (BDH), disodium hydrogen phosphate, sodium hydroxide (Merck) and phosphoric acid 85% (J.T. Baker) were used to prepare the buffer so-

lution. Phosphate buffer (0.1 M) solutions with pH ranges from 5.0 to 8.0, were prepared by dissolving the appropriate amounts of potassium dihydrogen phosphate and disodium hydrogen phosphate. Phosphoric acid and sodium hydroxide were used to adjust the desired pH that is out of the above range.

The standard tetracycline antibiotics were prepared by dissolving an appropriate amount of the tetracycline antibiotics in the buffer solution. The solutions were prepared daily.

2.2. Electrodes

The as-deposited BDD electrodes used in this work are from Professor Akira Fujishima's Laboratory. The electrodes were prepared by deposition of the BDD thin films on highly conductive *n*-Si(111) substrate using the technique of microwave plasma-assisted chemical vapor deposition. Deposition was usually carried out for 10 h to achieve a film thickness of approximately 30 μm. The nominal B/C atomic ratio in the gas phase was 1:100, and the typical boron-doped level in the film was ca. 10²¹ cm⁻³. The experimental conditions and the apparatus used for the diamond film growth have been described in detail elsewhere [23]. A piece of diamond was rinsed with ultrapure water prior to use for electroanalysis.

Anodized BDD electrode was prepared by treating an as-deposited BDD electrode in 0.1 M KOH solution. The potential was applied between 0 and 2.2 V versus Ag/AgCl using cyclic voltammetry for 30 min. The anodized BDD electrode was also rinsed with ultrapure water before use.

The GC electrode was purchased from Bioanalytical System, Inc. (area 0.07 cm²). It was pretreated by sequential polishing with 1 and 0.05 μm of alumina/water slurries on felt pads, followed by rinsing with ultrapure water prior to use.

2.3. Cyclic voltammetric investigation

Electrochemical experiments were conducted out in a single compartment three-electrode glass cell. The BDD electrode was pressed against a smooth ground joint at the bottom of the cell, isolated by an *O*-ring viton (area 0.07 cm²). Ohmic contact was made by placing the backside of the Si substrate. The GC carbon was used as working electrode for the comparison. The Ag/AgCl with salt bridge and Pt wire were used as reference electrode and counter electrode respectively. The voltammetric measurement was performed with the three types of electrodes using Autolab Potentiostat 100 (Eco-Chemie, The Netherlands).

2.4. Flow injection with amperometric detection

The flow injection system consisted of a thin layer flow cell (Bioanalytical System, Inc), a 20 mL stainless steel loop of injection port (Rheodyne 7725), a peristaltic pump, and

electrochemical detection (Autolab potentiostat 100). The carrier solution (0.1 M potassium dihydrogen phosphate pH 2) was regulated at the flow rate of 1 mL min^{-1} by measuring the volume of carrier solution in 1 min. The thin layer flow cell consisted of a silicone rubber gasket as a spacer, a Ag/AgCl electrode as the reference electrode and a stainless steel tube as an auxiliary electrode and an outlet of the flow cell. The experiments were performed in a copper faradaic cage to reduce the electronic noise. All experiments were done at room temperature.

2.5. Sample preparation

The contents within the pharmaceutical capsule (as described on the label) were: 250 mg tetracycline hydrochlorideTM (Tetracycline hydrochloride); 250 mg AureomycinTM (chlortetracycline); 250 mg OxycineTM (oxytetracycline); 100 mg MedomycinTM (doxycycline). The powder of twenty capsules of each drug was accurately weighed and homogenized so as to obtain the mean capsule weight. A portion of the homogenized powder corresponding to the mean capsule weight was then transferred into volumetric flask and dissolved in 0.1 M phosphate buffer (pH 2), and then mixed thoroughly. A portion of the sample solutions was filtrated through 0.45 mm nylon membrane. The filtrated solutions were further diluted using 0.1 M phosphate buffer (pH 2) to make the final concentration in the range of $0.96\text{--}5.15 \text{ mg mL}^{-1}$ within the linear dynamic range. All tetracycline antibiotic solutions were protected from light by covering with aluminum foil and stored at 4°C .

3. Results and discussion

3.1. Cyclic voltammetry

3.1.1. Voltammetric study

According to Fig. 1, the anodized BDD electrode (Fig. 1c) provided well-resolved irreversible oxidation cyclic voltammogram with the highest current responses in comparison to the as-deposited BDD and the glassy carbon electrodes, Fig. 1a and b respectively. These electrodes produced oxidative waves at $+1.0 \text{ V}$ versus Ag/AgCl, however the as-deposited BDD electrode produced no voltammetric peak shape in contrast to the results at the anodized BDD electrode. The results obtained from the other antibiotics, i.e., chlortetracycline, oxytetracycline and doxycycline were analogous to the results shown for tetracycline in Fig. 1. These results can be rationalized as follows. The surface of the BDD, after the anodizing treatment, is partly covered with oxygen atoms [30–32]. In acidic condition, all the tetracycline are positively charged. This therefore encourage electrostatic interaction between the negatively charged BDD anodized surface and the analytes. In contrast, the as-deposited BDD, the surface is non-polar (covered with hydrogen atoms), and therefore the electrostatic interaction

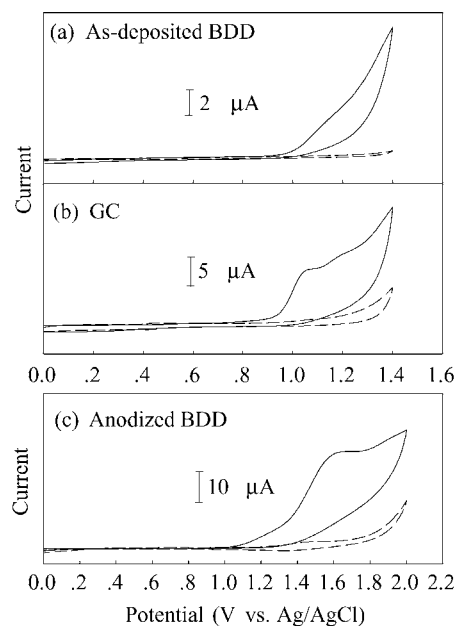


Fig. 1. Cyclic voltammetric results of $500 \mu\text{M}$ tetracycline (solid line) in 0.1 M potassium dihydrogen phosphate (pH 2) with the corresponding background current (dashed line) at: (a) as-deposited BDD; (b) glassy carbon; and (c) anodized BDD electrodes.

is less permitted. This may be the reason for the ill-defined peak current, in Fig. 1a, for example.

3.1.2. pH dependence

The effect of pH was investigated from pH 2–9 for all four compounds using anodized BDD electrode. A plot of pH versus E_p has been investigated. It was found that results were ambiguous, and the expected pK was inconsistent with the pK value of the dissociation in the literature.

For quantitative purposes, the information concerning about the current response was also investigated. It was found that in acidic buffer, tetracyclines provided the well-defined oxidative responses cyclic voltammograms. However, in neutral and alkaline buffer, ill-defined voltammetric waves were obtained. The current response decreased with increasing the pH. Results for all, the antibiotics demonstrated that pH 2 gave the highest current signal (Fig. 2). Therefore, pH 2 was chosen as the optimal pH for the rest of experiments.

It can be explained that tetracyclines are derived from a system of four membered rings arranged linearly with characteristic double bonds. They are amphoteric compounds with high polarity, and an isoelectric point between 4 and 6. From the literature [33,34], three dissociations have been observed for tetracyclines, with $pK_1 = 3.3$, $pK_2 = 7.5$ and $pK_3 = 9.4$. In strongly acidic pH, the tetracycline molecule exists in its fully protonated form as a singly charged cation. As proposed [30–32], the surface of anodized BDD electrode covered with the negative charge, electrostatically attracts the positive tetracycline. Hence, at pH 2 the positively charged tetracycline provided the highest response.

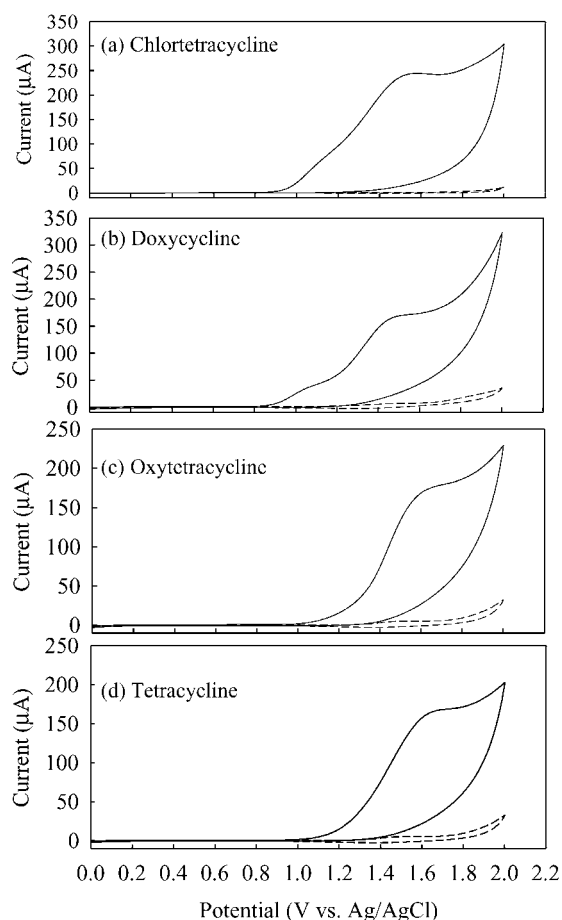


Fig. 2. Cyclic voltammograms at anodized BDD electrode vs. Ag/AgCl in 500 μ M chlortetracycline, doxycycline, oxytetracycline, and tetracycline in 0.1 M phosphate buffer pH 2 (solid lines) and 0.1 M phosphate buffer pH 2 (dashed lines). The scan rate was 50 mV s^{-1} ; area of electrode, 0.07 cm^2 .

Moreover, the anodized diamond surface can be recovered by in situ reactivation (applying a high positive potential 2.6 V versus Ag/AgCl) during the flow injection measurement [30].

3.1.3. Scan rate dependence

The scan rate dependence study was carried out by varying the scan rate ranging from 0.01 to 0.3 V s^{-1} . From the result, we found for all four compounds, that the relationship between current and square root of scan rate was linear with the correlation coefficient of approximately 0.99 (data not shown). The results indicated that the oxidation currents are controlled by the diffusion of tetracyclines in the interfacial reaction zone.

3.2. Flow injection with amperometric detection

3.2.1. Hydrodynamic voltammetry

Hydrodynamic voltammogram was obtained from the average of three injections at aliquot of 20 μ L of 100 mM tetracycline antibiotic solutions in the flow injection system. In

this case the applied potential was gradually increased from 1.1 to 2.0 V versus Ag/AgCl. The carrier solution was 0.1 M potassium dihydrogen phosphates (pH 2). Fig. 3a shows the hydrodynamic voltammogram of tetracycline antibiotics with the corresponding background currents. The hydrodynamic voltammogram of tetracycline antibiotics did not exhibit the sigmoidal shape of the signal versus potential probably due to the high oxidation potential of tetracycline. To obtain the optimum potential, we calculated the S/B ratios from the results in Fig. 3a at each potential and plotted the ratios versus potentials (Fig. 3b). The maximum S/B ratio was found at 1.5 V (versus Ag/AgCl) for chlortetracycline and at 1.6 V (versus Ag/AgCl) for tetracycline, oxytetracycline and doxycycline. Hence, these optimum potentials were used for the quantification experiments in amperometric flow injection.

3.2.2. Analytical performance of the FI system at anodized BDD electrode

A series of analytical features were performed on the proposed method for the quantification of tetracycline, chlortetracycline, oxytetracycline and doxycycline. Calibration parameters and the linear ranges for all the drugs are summarized in Table 1. The limit of detection (3S/N) was 10 nM for all compounds. To obtain the repeatability, 10 replicates of injections were carried out at three concentrations (5, 10, 50 mM). The peak variability defined as the relative standard deviation (R.S.D. %), were found to be 1.3–1.7 (for tetracycline hydrochloride), 2.4–3.0 for chlortetracycline, 1.6–3.0

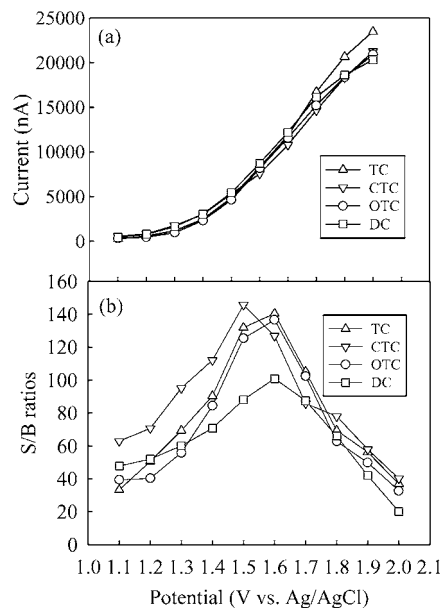


Fig. 3. Hydrodynamic voltammetric results for 100 μ M tetracycline antibiotics. (a) (Δ): tetracycline, TC; (∇): chlortetracycline, CTC; (\circ): oxytetracycline, OTC; and (\square): doxycycline, DC in 0.1 M potassium dihydrogen phosphate (pH 2), using 0.1 M potassium dihydrogen phosphate (pH 2) as carrier solution. The flow rate was 1 mL min^{-1} . (b) Hydrodynamic voltammogram of signal-to-background ratios.

Table 1

Regression parameters and linear range for the determination of tetracycline antibiotics by flow injection with amperometric detection using anodized boron-doped diamond electrode ($n = 2$)

Analytes	Linear range (μM)	a	b	r^2
Tetracycline	0.1–50	110.0 ± 5.9	192.1 ± 5.0	0.997
Chlortetracycline	0.5–50	76.9 ± 3.9	199.7 ± 29.7	0.997
Oxytetracycline	0.5–50	90.2 ± 3.0	165.2 ± 28.9	0.999
Doxytetracycline	0.5–50	96.2 ± 3.8	213.7 ± 48.9	0.997

$ay = ax + b$, where y : current (nA); x : concentration (μM); a : slope (nA/ μM); b : intercept (nA).

Table 2

Determination of tetracycline antibiotics in drug formulations ($n = 2$)

Drug formulations	Label amount (mg)	Found (mg)	Found (%)
Tetracycline	250	249.4 ± 1.7	99.8 ± 0.7
Aureomycine	250	249.7 ± 1.3	99.87 ± 0.5
Oxycine	250	254.6 ± 2.5	101.85 ± 1.0
Medeomycin	100	98.4 ± 1.0	98.4 ± 1.0

for oxytetracycline and 1.7–2.7 for doxycycline. The stability of the amperometric response using flow injection at the anodized BDD electrode was obtained by conducting 100 repetitive injections of 50 mM chlortetracycline. It was observed that the peak variation was about 3.4%. The throughput of sample is approximately seventy injections per hour.

3.2.3. Application

Our method was applied for determination of each compound in four drug formulations using the standard addition method. The results are presented in Table 2. The percentages of recovery indicate that the amounts are comparable between the labeled quantity and the value obtained from the proposed method.

4. Conclusion

Anodized treatment has significantly improved the analytical performance of the BDD thin film for the analysis of tetracycline drugs. Current signal of the anodized BDD electrode is greater than the current signal of the glassy carbon electrode. It was observed from the cyclic voltammetric study that pH strongly influenced the current response of the voltammograms with pH 2 being selected for the method development, since the highest current was obtained with a well-defined voltammetric signal.

Amperometric detections of four compounds, i.e., tetracycline, chlortetracycline, oxytetracycline and doxycycline was employed in the method development, using flow injection. The developed method has been successfully applied to determine four types of tetracyclines in various pharmaceutical formulations. The tetracycline antibiotics content of the drug was found to be in excellent agreement with the declared amount on the label.

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