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Short communication

Voltammetric behavior of benznidazole at a DNA-electrochemical biosensor

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Abstract

Benznidazole is a drug used commonly as a therapeutic agent against Chagas' disease in Brazil. To clarify the cytotoxic action of benznidazole the electrochemical reduction of benznidazole has been investigated using a DNA-electrochemical biosensor, prepared by modification of a glassy carbon electrode with DNA, and the results compared with reduction at a bare glassy carbon electrode. The dependence of peak potential with pH follows slopes of 59 and 52 mV per pH unit in acid media, respectively, which corresponds to a mechanism involving the same number of electrons and protons. In neutral and alkaline solution no significant dependence of peak potential with pH was found. During the electrochemical reduction of benznidazole the formation of the hydroxylamine derivative occurs, involving a total of four electrons. The potentials for reduction were less negative when using the DNA-modified glassy carbon electrode than at the bare glassy carbon electrode although the mechanism was the same, and at pH 7.51 the peak current was four times higher than that obtained with the bare electrode. The DNA-biosensor enabled pre-concentration of the drug onto the electrode surface and the in situ damage caused to the DNA on the electrode surface by the product of benznidazole reduction could be detected electrochemically. The results are in agreement with the hypothesis that the hydroxylamine derivative is the reactive species responsible for the cytotoxic action of benznidazole.

Keywords: Benznidazole; DNA-modified electrode; Action mechanism; DNA-biosensor

1. Introduction

Nitroimidazoles are compounds that have been used in therapeutics during the last 40 years. They are important because they are wide-spectrum drugs, being active against Gram-positive and Gram-negative bacteria, protozoa, helmintes, hypoxic tumours, and causing a low level of resistance in anaerobic microorganisms [1–4].

The first nitroimidazole with biological activity investigated was metronidazole, showing preferen-

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tial action on *Trycomoniasis vaginalis* [5], and since then studies on other nitroimidazoles have appeared [1–3]. Benznidazole (BZN) [5,6], is commonly used for Chagas' disease treatment, being the only drug marketed in Brazil [7] against this parasitosis.

Chagas' disease affects about one quarter of Latin America's population, about 100 million people living under the risk of contracting the parasitosis and 16–18 million people are infected according to the World Health Organization (WHO) [8]. In Brazil, where about 6 million people are infected, the main problem is resistance of *T. cruzi* to nifurtimox the other anti-trypanosomal drug available. Both benznidazole and nifurtimox are only effective in the acute phase of the disease [7].

The biological action of nitrocompounds is dependent on the reduction of the nitro group. The nitro anionic radical and hydroxylamine derivative are the main intermediate products responsible for the cytotoxic action of the nitroimidazoles [3,6,9]. The pharmacological properties of these compounds have been quantitatively related to reduction of the redox couple $R-NO_2/R-NO_2^{\bullet -}$ and hydroxylamine formation [9,10].

Structure–activity relationship studies [11] demonstrated that the reduction potential correlates with the antimicrobial activity of nitro-heterocyclic compounds. Some of these compounds have tripanomicidal action because the flavoproteins are able to reduce the nitrocompound to the nitroradical or because of the formation of superoxide and hydrogen peroxide, as a consequence of electron transfer from the nitroradical to the molecular oxygen [3,4,12].

Experiments demonstrate that the products of nitro group reduction react with nucleic acids in vitro and it is believed that DNA is the main target in vivo [13]. Both double helix and single-stranded DNA suffer the action of nitroimidazole drugs [14]; the damage to DNA depends on the stability of the intermediate product formed on drug reduction. The nitro anionic radical is the species principally responsible for DNA damage, oxidizing the DNA double helix and inducing thymidine phosphate release [1,3]. On the other hand, it was shown that 5-hydroxylamineimidazole could be the species principally responsible for the bactericide action of the nitroimidazole [15]. However, for 2-nitroimidazoles, such as benznidazole, extensive formation of guanine adducts across the phosphate-ester link is considered, resulting from reduction of the drug and hydroxylamine derivative formation [16].

The nucleic acid bases interact with the intermediate generated during the nitroimidazole reduction [1,3,13], with preferential nitroimidazole–guanine interaction [13]. 5-Nitroimidazole, metronidazole, tinidazole and secnidazole, were studied using a DNA-biosensor, prepared by modification of a glassy carbon electrode with DNA [17,18]. It was possible to pre-concentrate the drugs in the DNA layer physisorbed on the electrode surface, which is a great advantage of the biosensor. With pre-concentration, the voltammogram for the cathodic peak of metronidazole was a high, sharp and symmetric peak, corresponding to reduction of the nitro group to the hydroxylamine derivative. This hydroxylamine formed by reduction of nitroimidazoles causes in situ damage to the DNA on the electrode surface, which is electrochemically detected. In these experimental conditions a low detection limit of 1.0 μM was obtained for metronidazole [20,21] and this methodology showed good possibilities for comprehension of the biological mechanism of nitroimidazoles.

The voltammetric reduction of 5-nitroimidazoles using mercury [19] and solid [20,21] electrodes has been investigated. The mechanism of action of nitroimidazole as antimicrobial agent [11], and for the analysis of pharmaceutical [22,23] and clinical samples [22,24] was also studied voltammetrically. However, the only published electrochemical study of benznidazole, a 2-nitroimidazole, concerns its polarographic determination in dimethylsulfoxide [25].

In this paper the voltammetric behaviour of benznidazole at a glassy carbon electrode and at a DNA-biosensor is presented. The study of the benznidazole–DNA interaction after in situ electrochemical generation of the hydroxylamine derivative which damages the immobilized DNA on the glassy carbon electrode surface was possible using the DNA-biosensor.

2. Experimental

2.1. Chemicals and solutions

Benznidazole (BZN) was supplied by Roche Farmacêutica, Brasil. A 0.01 M solution of benznidazole was prepared by direct dissolution in ethanol 80% and kept at 4 °C. No chemical changes of the analyte solution were observed during the experiments.

Calf thymus DNA (sodium salt, type I), was obtained from Sigma Chemical Co. and was used without further purification. Single stranded DNA (ss DNA) was prepared by treating an accurately weighed sample of approximately 3 mg of DNA with 0.5 ml of 60% pure perchloric acid; after dissolution 0.5 ml of 9M NaOH was added to neutralize the solution. The volume was completed to 10 ml with 0.1 M pH 4.5 acetate buffer and the solution kept at 4 °C.

The pH study was performed using universal buffer solutions prepared from a mixture of phosphoric acid, acetic acid and boric acid with NaOH [26]. Acetate buffer pH 4.5 with ionic strength 0.1 M was also used as supporting electrolyte. All reagents were from Merck and the solutions were prepared using purified water from a Millipore Milli-Q system (conductivity < 0.1 S cm^{-1}).

All experiments were carried out at room temperature and in deoxygenated solution, after bubbling nitrogen for 10 min.

2.2. Apparatus

Voltammograms were recorded using a μAutolab potentiostat/galvanostat running with GPES version 4.1 software, from Eco-Chemie, Utrecht, Netherlands. Differential pulse voltammetry conditions were: pulse amplitude 50 mV, pulse width 70 ms and scan rate 5 mV s^{-1}. All experiments were performed using an electrochemical cell of 10 ml with a three electrode system consisting of a glassy carbon or DNA-modified glassy carbon electrode as working electrode ($d = 3.0$ mm), a platinum auxiliary electrode and a Ag/AgCl reference electrode. Differential pulse voltammograms were recorded using a scan rate of 5 mV s^{-1}, pulse amplitude 50 mV and pulse width 70 ms, while cyclic voltammograms were recorded at scan rate of 100 mV s^{-1}.

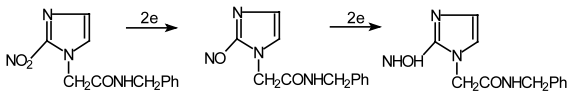
A Crison micropH 2001 pH meter in conjunction with a combined glass electrode from Ingold was used for pH measurement.

2.3. DNA-biosensor

The DNA-biosensor was prepared by covering the glassy carbon electrode with 3 mg of native DNA dissolved in 80 μl of pH 4.5 acetate buffer. This DNA-modified glassy carbon electrode was then left to dry. The DNA-modified glassy carbon electrode was placed in the electrochemical cell containing acetate buffer (pH 4.5) and conditioned at $+1.4$ V during 5 min. Afterwards it was immersed in a 78 μg ml^{-1} ssDNA solution and several differential pulse voltammograms were recorded between 0 and $+1.4$ V versus SCE to check that no electrochemical reaction occurs on the surface of the DNA-modified glassy carbon electrode in supporting electrolyte. This conditioning process was repeated until stable peak currents were obtained for guanine and adenine oxidation. At this stage the DNA-biosensor reached the maximum activity for electroanalytical applications [27], and was then dried at room temperature and used to study the mechanism of action of nitroimidazoles with DNA. The DNA-biosensor prepared in this way has electroanalytical stability during 4 days of constant use.

3. Results and discussion

The electrochemical reduction of nitroimidazole compounds follows a complex mechanism. Theoretically, the nitro group can receive up to six electrons in the complete reduction to the amine derivative [1,3,19]. Voltammetric nitroimidazole reduction indicated nitroso (R–NO) and hydroxylamine (R–NHOH) derivative formation, involving a total of four electrons, as shown in Scheme 1 [19].



Scheme 1. Mechanism of reduction of benznidazole.

3.1. Benznidazole reduction

Differential pulse voltammetry was used to study the reduction of benznidazole at two different pH values, 3.06 and 7.51, using a bare glassy carbon electrode (curve 1) and a DNA-biosensor (curve 2) as shown in Fig. 1. For both pHs only one reduction wave was obtained, corresponding to reduction of the nitro group to the hydroxylamine derivative. The peak potential using the DNA-biosensor occurs at potentials 40 and 30 mV less negative than observed at the bare glassy carbon electrode for pH 3.06 and 7.51, respectively. Additionally, at pH 7.51 the peak current obtained at this DNA-modified glassy carbon electrode was four times higher than that obtained with the bare glassy carbon electrode.

In a similar way to what was observed for nitroimidazole [20,21], benznidazole reduction is also pH-dependent in acidic medium, the reduction peak potential being shifted to negative values with increasing pH. The variation of E_p for the reduction of benznidazole with pH, Fig. 2, shows slopes of 59 and 52 mV per pH unit, respectively, with the DNA-modified glassy carbon electrode and bare glassy carbon electrode. This behaviour confirms proton participation in the reduction process of benznidazole, and corresponds to a mechanism involving the same number of electrons and protons. For pH values above 7.0, no significant variation of E_p with pH was observed and the values obtained for the peak potentials with both types of electrode were very close. The main reduction product is the hydroxylamine derivative, as for metronidazole [20,21].

For pH > 7.0 the peak current values for the reduction wave of benznidazole were also shifted, but no significant linear relationship was observed. Using voltammetry the nitroimidazoles can be reduced in only one step to the amine derivative in alkaline medium. Under these condi-

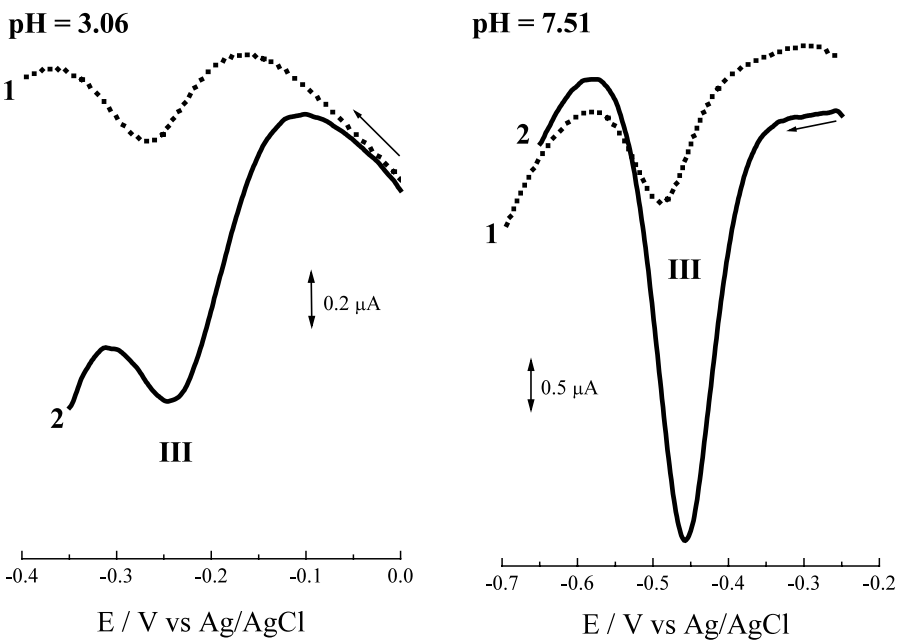


Fig. 1. Differential pulse voltammograms of 50.0 mM benznidazole in universal buffer: (1) glassy carbon electrode; (2) DNA-modified glassy carbon electrode. Scan rate 5 m V s^{-1}.

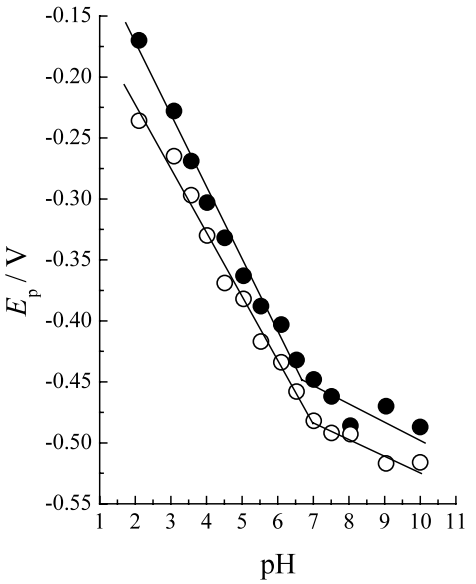


Fig. 2. Plot of E_p vs. pH for a solution of 50.0 mM: benznidazole (●) DNA- modified glassy carbon electrode; (○) glassy carbon electrode.

tions it was not possible to distinguish the full six-electron reduction of benznidazole [25].

The results obtained for the benznidazole reduction using a DNA-biosensor demostrate good possibilities for developing an electronalytical methodology for benznidazole determination. However, in the case of this work the objective was the use of the voltammetric methods for the study of the biological mechanism of benznidazole–DNA interaction and of the structure–activity relationship, which can be an important parameter in structure–activity studies.

3.2. Benznidazole–DNA interaction

The reduction mechanism using the DNA-biosensor or the bare glassy carbon electrode is the same. However, the significant increase in peak current due to pre-concentration of the DNA matrix on the electrode surface of benznidazole enables larger peak currents to be obtained and thus permits more easily the study of DNA–benznidazole interaction.

For the purpose of studying DNA-benznidazole interaction the procedure was as follows. During the experiments benznidazole was first pre-concentrated from bulk solution on the DNA multilayer immobilised on the glassy carbon surface, i.e. the DNA-biosensor. The pre-concentration concentration conditions were 5 min deposition time, $t_d = 5$ min, at an applied fixed deposition potential, $E_d = -0.4$ V. According to the mechanism of reduction of benznidazole at this potential on the electrode surface in situ formation of the hydroxylamine derivative occurs, Fig. 1, which reacts with the DNA in the biosensor and is responsible for the damage caused to DNA. The deposition time used has already been optimised [17].

The DNA–benznidazole interaction was followed by cyclic and differential pulse voltammetry.

The cyclic voltammograms, Fig. 3, show four very well-defined peaks: $E_{p,Ia} = +0.26$ V; $E_{p,Ic} = +0.17$ V; $E_{p,II} = +0.60$ V and $E_{p,III} = -0.37$ V. The peaks $\mathbf{I}_a$ and $\mathbf{I}_c$ can be attributed to the reversible hydroxylamine/nitroso derivative couple [17,18], product of voltammetric reduction of benznidazole, according to the chemical equilibrium

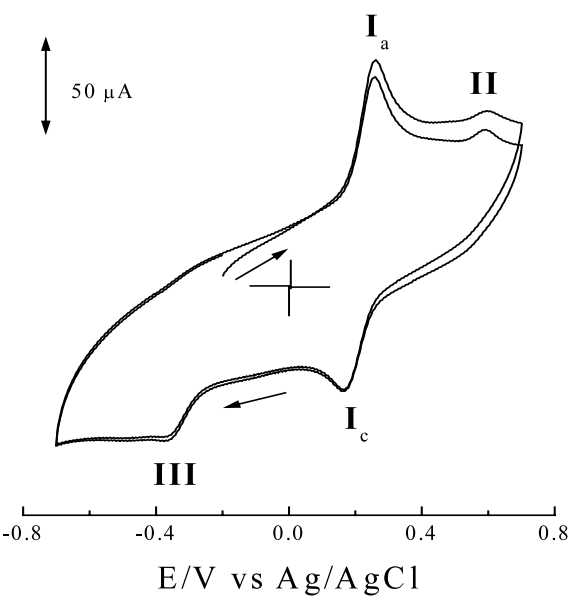


Fig. 3. Cyclic voltammogram of 0.43 mM benznidazole obtained with the DNA-modified glassy carbon electrode in pH 4.5 0.1 M acetate buffer solution. Scan rate 100 mV s^{-1}, $E_d = -0.4$ V, $t_d = 5$ min and $E_i = -0.2$ V.

$$R-NHOH \underset{I_c}{\overset{I_a}{\rightleftarrows}} R-NO+2e+2H^+$$

Peak **II** can be identified as the possible interaction between the benznidazole reduction products and DNA immobilised on the electrode surface. The characterisation of this peak was based on previous results [17,18] and on literature data that presents the purine bases [13] as the main target of nitroimidazole drugs for oxidative damage to DNA. According to Dryhust [28], oxidation products of purine bases have anodic peaks around 0.5 V. Based on his findings, peak **II** would correspond to deoxypurinic acid oxidation, for example 8-oxoguanine [29]. The deoxypurinic acid is formed as a consequence of the action of the products of benznidazole reduction on the DNA immobilised on the electrode surface. Peak **III** corresponds to the four electron benznidazole reduction studied in the previous section.

The benznidazole–DNA interaction was more fully investigated by differential pulse voltammetry. Differential pulse voltammograms, Fig. 4, show two very well-defined peaks: $E_{p,Ia} = +0.20$ V and $E_{p,II} = +0.53$ V and several smaller oxidation peaks, the size of which increase with benznidazole concentration.

Because the voltammetric behaviour of nitroimidazoles and their interactions with DNA bases had previously been studied using mercury electrodes [13,19,25,30], it was not possible due to the mercury electrode to investigate the anodic region. The use of the DNA-biosensor allowed the anodic voltammetric study of nitroimidazoles and led to an explanation for the differences in reactivity between 5-nitroimidazoles and 2-nitroimidazoles. The results obtained with benznidazole are quite different to those obtained with metronidazole [17,18]. In the latter, a well-defined peak was observed at $+0.224$ V and attributed to an azoxycompound formed during the condensation reaction between hydroxylamine and its nitroso derivative at the electrode surface. The amount of azoxycompound formed depends on the concentration of the nitroso derivative, which is reduced rapidly to hydroxylamine [17]. Nevertheless, formation of this compound was not observed during the anodic scan of benznidazole. It seems that the hydroxylamine derivative-DNA interaction

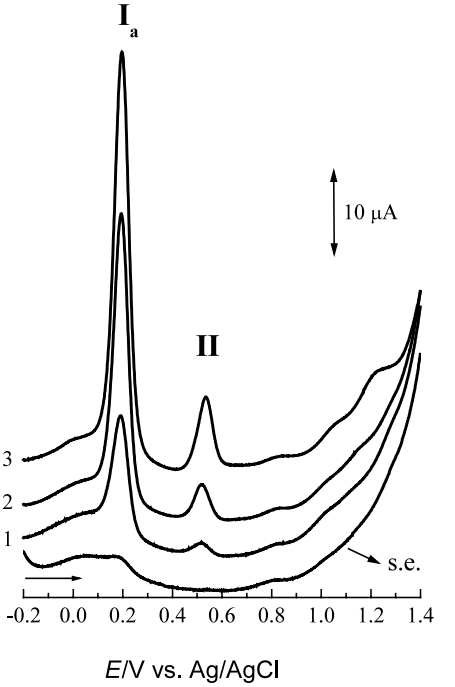


Fig. 4. Differential pulse voltammograms of benznidazole obtained with the DNA-modified glassy carbon electrode in pH 4.5 0.1 M acetate buffer: 1, 0.050 mM; 2, 1.50 mM; 3, 43.0 mM; s.e.—supporting electrolyte. Scan rate 5 mV s^{-1}, $E_d = -0.4$ V, $t_d = 5$ min and $E_i = -0.2$ V.

was sufficiently fast and efficient to avoid the condensation reaction. This result corroborates the hypothesis that the hydroxylamine derivatives of 2-nitroimidazoles are the reactive species responsible for cytotoxic drug action [15,16].

The smaller oxidation peaks can be identified with the oxidation of the purine nucleotides guanosine ($E_{p,a} = +1.05$ V) and adenosine ($E_{p,a} = +1.20$ V), caused by the DNA–benznidazole interaction.

The effect of drug concentration on the peak currents of $E_{p,I}$ and $E_{p,II}$ is shown in Fig. 5. Standard addition of benznidazole caused an increase in current values for the two peaks. The current for peak **I**, corresponding to hydroxylamine oxidation, reaches a plateau. This can be explained by saturation of the DNA immobilised on the glassy carbon electrode. For the second peak, the current varies linearly with benznidazole concentration, in spite of a less significant increase of current with concentration increment.

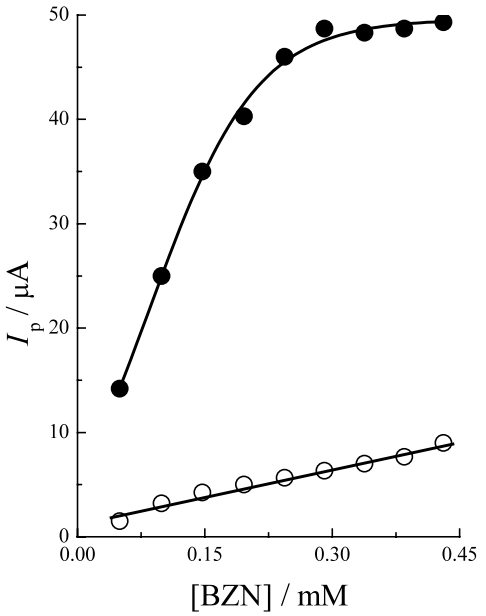


Fig. 5. Standard addition curves of benznidazole from differential pulse voltammograms obtained with the DNA-modified glassy carbon electrode in pH 4.5 0.1 M acetate buffer: (●) peak **I**$_a$ ($E_p = +0.18$ V); (○) peak **II** ($E_p = +0.55$ V). Scan rate 5 m V s^{-1}, $E_d = -0.4$ V, $t_d = 5$ min and $E_i = -0.2$ V.

The use of the DNA-biosensor for the understanding of DNA interactions with molecules or ions exploits the application of voltammetric techniques for in situ generation of reactive intermediates. It is a complementary tool for the study of biomolecular interaction mechanisms of drugs, such as benznidazole–DNA, showing an interesting and interdisciplinary approach between analytical and medicinal chemistry.

4. Conclusions

The DNA-biosensor was used in the present work and clearly shows the first electrochemical results for in situ sensing of DNA damage caused by reduced benznidazole and the possibility of pre-screening the damage caused to DNA integrity. Knowledge of the benznidazole–DNA interaction mechanism will be of great importance for establishing models for the development of new drugs for Chagas' disease.

These results also aid in understanding the mechanism of action of nitroimidazoles and explain the differences in reactivity between different nitroimidazoles. They can be used as important parameters for quantitative studies of structure–activity relationships (QSAR), and as a contribution to the design of new candidates to drugs.

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