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Voltammetry of the interaction of metronidazole with DNA and its analytical applications

Xiaohua Jiang, Xiangqin Lin *

Department of Chemistry, University of Science and Technology of China, Hefei 230026, People's Republic of China

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Abstract

Voltammetric methods were used to probe the interaction of antimicrobial drug metronidazole (MTZ) with calf thymus DNA. Binding constants (K) and binding site sizes (s) were determined from the voltammetric data, i.e., shifts in potential and changes in limiting current with the addition of DNA. MTZ showed appreciable electrostatic binding to DNA in solution with $K = 2.2(\pm 1.3) \times 10^4$ M^{-1} and $s = 0.34$ bp. One reduction peak of MTZ at the bare glassy carbon electrode (GCE) split into two peaks at the DNA modified GCE (DNA/GCE). These changes in the cyclic voltammogram can only be due to the interaction of MTZ with the surface-confined DNA. In addition, the peak current of MTZ at the DNA/GCE was nearly 8-fold of the response at the bare GCE. The low detection limit of 2.0×10^{-8} M made the DNA/GCE a promising biosensor for MTZ determination. And this method was successfully applied with high precision and accuracy compared with spectroscopic methods (relative error <6%) for estimation of the total MTZ drug content in pharmaceutical dosage forms.

Keywords: Drug analysis; Metronidazole; Interaction; DNA; Voltammetry; Biosensor

1. Introduction

Deoxyribonucleic acid (DNA) plays a central role in life processes since it contains all of the genetic information required for cellular function. However, DNA molecules are prone to be damaged under various conditions, especially by interaction with some molecules, and this damage may lead to various pathological changes in living organisms. There is growing interest in exploring the binding of small molecules with DNA for the rational design and construction of new and more efficient drugs targeted to DNA as well as in understanding how proteins recognize and bind to specific DNA sequences [1–3]. The specific interaction between a drug and DNA results in some changes in physical and chemical properties and has a potential importance for a better understanding of its therapeutic efficiency, which may also be used for conformational recognition to find new structures of DNA [4–6]. Various techniques have been extensively employed to study the interaction of these molecules with DNA, such as spectroscopic methods [7–9], isothermal calorimetric titration [10], nuclear magnetic resonance [11], quartz crystal microgravimetry [12], etc. Since many small molecules exhibit redox activity, electrochemical method should provide a useful complement to the previously used methods for investigation [13–16]. Moreover, the electrochemical behaviors are valuable in elucidating some mechanisms of the drug action in vivo [17,18]. Although the binding modes of several molecules with large conjugated structures have been delineated, much less attention was paid to the binding of simple heterocyclic compounds.

5-Nitroimidazoles are a well-established group of antiprotozoan and antibacterial agents [19]. They have a heterocyclic structure consisting of an imidazole-based nucleus with a nitro group, NO_2, in position 5. Based on the bioreduction of the nitro group and the generation of short-lived reactive intermediates which exhibit cytotoxicity towards cellular systems, these chemotherapeutic agents inhibit the growth of both anaerobic bacteria and certain anaerobic protozoa, such as *Trichomonas vaginalis*, *Entamoeba histolytica* and *Giardia lamblia*. Besides, they are also used as hypoxic cell cytotoxins and radiation sensitizers for continuing investigation in the treatment of cancer. One representative of the cytostatic drugs

* Corresponding author. Tel.: +86 0551 3606646; fax: +86 0551 3601592.
E-mail address: xqlin@ustc.edu.cn (X. Lin).

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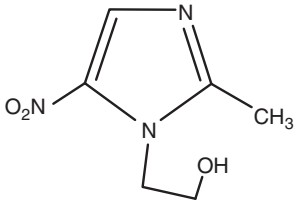

Fig. 1. The structure of MTZ molecule.

is metronidazole (MTZ), 1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole (Fig. 1), the short-lived reduced substrates of which are cytotoxic and interact with DNA to cause a loss of helical structure and strand breakage resulting in inhibition of nucleic acid synthesis and cell death [20–23].

In view of the similarity between the electrochemical reactions at the electrode/solution interface and the enzymatic redox reactions, knowledge of the electrochemical mechanism of reduction of MTZ would be very useful in providing additional information on its pharmacological activity [24]. Several works on electroreduction of MTZ at DNA modified electrode have been published [25–27]. However, no specific information about the interaction of MTZ with DNA was obtained and these methods was not sensitive with detection limits of only 10^{-6} M MTZ. Re-investigation has been carried out for the DNA-MTZ interaction by electrochemically grafting the DNA on a glassy carbon electrode surface, which is denoted as DNA/GCE. Interestingly, the reduction of MTZ at this DNA modified electrode gives a reversible one-electron redox reaction corresponding to nitro/nitro radical anion processes, and a successive irreversible reduction wave corresponding to a three-electron and four-proton reduction of nitro radical anion to hydroxylamine derivatives. Some valuable parameters, i.e., the diffusion coefficients for both of free and binding MTZ (D_f, D_b), the binding constant (K) and the binding site size (s, bp) of MTZ at the DNA were determined. It is obvious that MTZ on the DNA/GCE shows a different mechanism of their interaction. Additionally, the DNA/GCE is a promising biosensor for MTZ determination with a detection limit of 2.0×10^{-8} M.

2. Experimental

2.1. Materials

Calf thymus DNA (CTDNA) was obtained from Sino-American Biotechnical Co. and used as received. Solutions of DNA was 50 mM NaCl+5 mM Tris buffer (THB), pH 7.1 and gave rations of UV absorbance at 260 and 280 nm (A_{260}/A_{280}) of approximately 1.9, indicating that the CTDNA was sufficiently protein-free. The concentration of DNA solution, expressed in M of nucleotide phosphate (NP), was determined by UV absorbance at 260 nm using the molar extinction coefficient (ε) of 6600 M^{-1} cm^{-1} [28]. MTZ was purchased from Sigma. The supporting electrolyte was a Britton–Robinson (BR) buffer prepared in the usual way, by adding appropriate amounts of sodium hydroxide (0.2 M) to an orthophosphoric acid, acetic acid and boric acid mixture (0.04 M each). Double-distilled water was used to prepare the solutions. All other reagents were of analytical grade.

2.2. Instrumentation

Voltammetric experiments were performed on CHI 660A workstation (CHI, USA) with a conventional three-electrode system which consisted of a saturated calomel reference electrode (SCE), a Pt foil auxiliary electrode, and a working electrode. For voltammetric measurements, the test solution was placed in a single-compartment cell of volume 5–10 mL. A glassy carbon electrode (GCE) with a geometric area of 0.126 cm^2 was used for electrochemical measurements barely or after surface modification. Prior to experiments, the GCE was polished with 0.25-μm diamond paste on a nylon buffing pad and then subjected to ultrasonic cleaning in ethanol for 10 min. Absorption spectra were measured on a UV-2401 PC spectrophotometer, Shimadzu Corp., Japan. A quartz cuvette of 1.0 cm path length was used (Lightpath Optical Ltd., UK).

2.3. Procedures

To study the interaction of MTZ with solution-phase DNA, both voltammetric and spectroscopic titrations were carried out in pH 7.0 BR solutions. Keeping both the concentration of MTZ and the volume of solution constant, the electrochemical and absorption spectroscopic measurements were carried out for monitoring the system while varying the CTDNA concentration (titration). Solutions were deoxygenated via purging N_2 gas for 15 min before experiment and keeping N_2 atmosphere throughout the measurement.

A DNA/GCE was prepared for the study of interactions of MTZ with surface-linked DNA. A freshly prepared bare GCE was dipped into a 1.7×10^{-4} M NP solution under an applied electrode potential of + 1.7 V vs. SCE for 30 min for DNA immobilization. The obtained DNA/GCE was cleaned then transferred to a MTZ solution for voltammetric measurements. The pH effect was performed in BR buffer from pH 3.0 to pH 12.0.

Three kinds of pharmaceutical formulations, i.e. tablet, suppository and injection were employed to evaluate MTZ contents. The average mass of five tablets was determined and the tablets were finely powdered, and then a required amount of the sample was transferred into a 100-mL standard flask containing 80 mL of BR buffer (pH 9.0). The contents of flask were stirred magnetically for 15 min and then diluted to the volume with the buffer for obtaining ca. 10^{-3} M MTZ solution. The solution was filtered and the first 20 mL of the filtrate was discarded. Appropriate solutions were prepared by taking suitable aliquots of the filtrate and diluting with the buffer. No pretreatment for the injection was needed except for dilution with the buffer solution. Voltammograms were monitored at the DNA/GCE as in standard MTZ solutions. The content of MTZ was calculated from the regression equation.

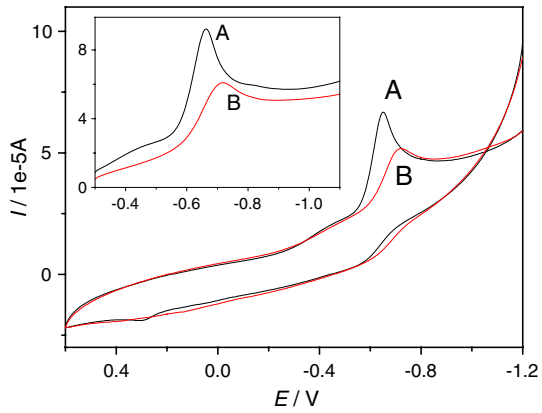

Fig. 2. Cyclic and linear sweep (inset) voltammetry of 0.5 mM MTZ in the (A) absence and (B) presence of 200.8 μM NP. Scan rate=50 mV/s.

All experiments were carried out at the ambient temperature (20–25 °C). All data, unless specified otherwise, are the average of at least three to five replicate measurements.

The Origin 7.0 software was used for linear regression analysis of I_p vs. $v^{1/2}$, E_p vs. log v and I_p vs. C plots. Nonlinear regression analysis of titration data was based on the deduced electrochemical equation.

3. Results and discussion

3.1. Interaction of MTZ with CTDNA in solution

The electrochemical behaviors of MTZ in the absence and in the presence of CTDNA at bare GCE were shown in Fig. 2 by either cyclic voltammetry (CV) or linear sweep voltammetry (LSV). A single CV reduction peak of MTZ was observed at −0.662 V vs. SCE in pH 7.0 BR buffer. The absence of any peaks in the reverse scan as well as the shift of the peak potential to negative direction on increasing the scan rate (see below) revealed the irreversible nature of the reduction.

The addition of CTDNA into the MTZ solution caused a considerable diminution in peak current and a large negative shift in peak potential, as shown in Fig. 2, line B. The binding of MTZ to CTDNA should lead to a significant decrease of

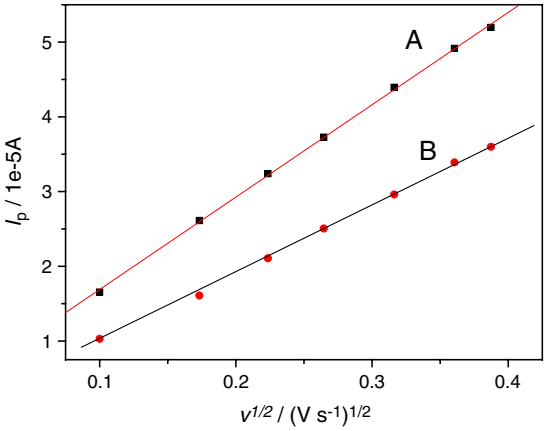

Fig. 3. Relationship between I_p and $v^{1/2}$ for 0.5 mM MTZ in the (A) absence and (B) presence of 200.8 μM NP by linear sweep voltammetry.

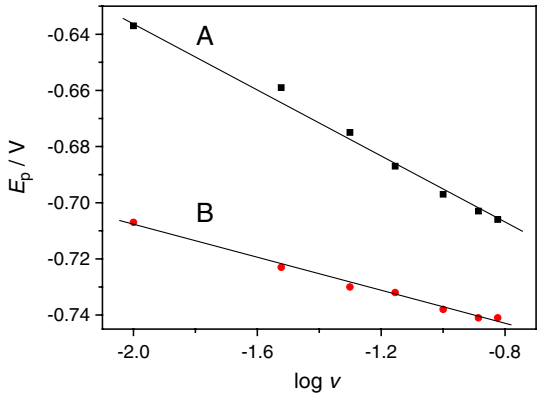

Fig. 4. Relationship between E_p and log v for 0.5 mM MTZ in the (A) absence and (B) presence of 200.8 μM NP by linear sweep voltammetry.

MTZ peak current due to the formation of MTZ-CTDNA adduct with very small diffusion coefficient [29,30]. This is obvious from the significant decrease in the slope of linear I_p–$v^{1/2}$ plots (R>0.9992) (Fig. 3). The slope was 1.23×10^{-4} A s$^{1/2}$ V$^{-1/2}$ in the absence of CTDNA (curve A) but and 9.11×10^{-5} A s$^{1/2}$ V$^{-1/2}$ in the presence of 200.8 μM CTDNA in NP. For the irreversible redox reaction of MTZ, an αn_a value (α, the electron transfer coefficient; n_a, the number of electrons involved in the rate-determining step) can be evaluated as 0.50 and 1.02 for free MTZ and MTZ-CTDNA adduct based on the slope of the E_p versus log(v) plot (Fig. 4). From these values, a diffusion coefficient (D_f) of the free MTZ was found to be 5.4×10^{-6} cm^2 s^{-1}, whereas $D_b=1.4\times10^{-6}$ cm^2 s^{-1} for the bound MTZ-CTDNA. These values are within the range reported by Carter et al. [13]. It was noteworthy that the CTDNA (200.8 μM NP) did not exhibit any Faradic responses at potentials between 0.6 and −1.2 V under our experimental condition. A −54 mV shift in peak potential (E_p) for MTZ was measured as the difference between the E_p at [NP]=0 and an averaged E_p value at the plateau of the $E_p\sim$[NP] curve (Fig. 5A). The negative shift of E_p indicates the oxidized form was bound on the DNA more strongly than its neutral form [13].

For an irreversible reaction at 25 °C, the peak current (I_p) of MTZ can be calculated [31]:

$$I_p = B\left[(\alpha n_a)_f^{1/2} D_f^{1/2} C_f + (\alpha n_a)_b^{1/2} D_b^{1/2} C_b\right] \quad (1)$$

where $B=2.99\times10^5 nAv^{1/2}$ [32], A is the surface area of the working electrode, C_b and C_f represents the equilibrium concentration of MTZ (bonded in MTZ-DNA) and the concentration of free MTZ, and the total concentration of MTZ, C_t, is

$$C_t = C_f + C_b. \quad (2)$$

Based on Carter et al. [13], the binding constant, K, can be expressed as the following form:

$$K = \frac{C_b}{C_f\left(\frac{[NP]}{2s} - C_b\right)} \quad (3)$$

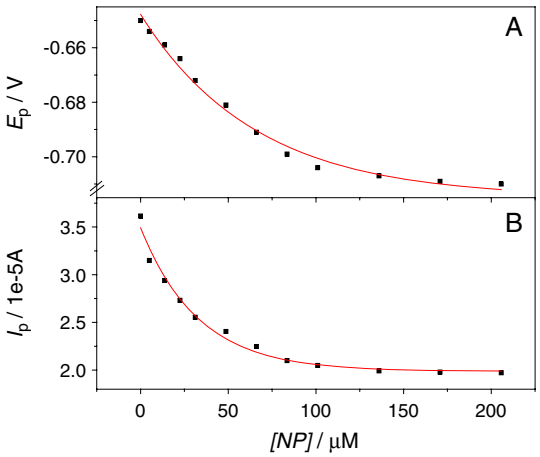

Fig. 5. Dependence of E_p (A) and I_p (B) of 0.5 mM MTZ on the concentration of added NP by cyclic voltammetry. Scan rate = 50 mV/s.

where s is the size of binding site in terms of bp. Making appropriate substitutions and eliminating C_b and C_f from Eq. (1), a new equation was obtained:

$$I_p = B\left\{(\alpha n_a)_f^{1/2} D_f^{1/2} C_t + \left[(\alpha n_a)_b^{1/2} D_b^{1/2} - (\alpha n_a)_f^{1/2} D_f^{1/2}\right] \times \left[\frac{b - \left(b^2 - \frac{2K^2 C_t [NP]}{s}\right)^{1/2}}{2K}\right]\right\} \quad (4)$$

where $b = 1 + KC_t + K[NP]/2s$. Since I_p, C_t and [NP] are experimentally measurable and $(\alpha n_a)_f$, $(\alpha n_a)_b$, D_f and D_b have already been acquired as mentioned above, the binding constant (K) and binding site size (s) of the MTZ-DNA can be obtained from a nonlinear regression analysis of the experimental data ($I_p \sim$ [NP] plot) according to Eq. (4). Fig. 5 showed the results for voltammetric titration of 0.5 mM MTZ with CTDNA. For the binding curve B, a nonlinear fit analysis

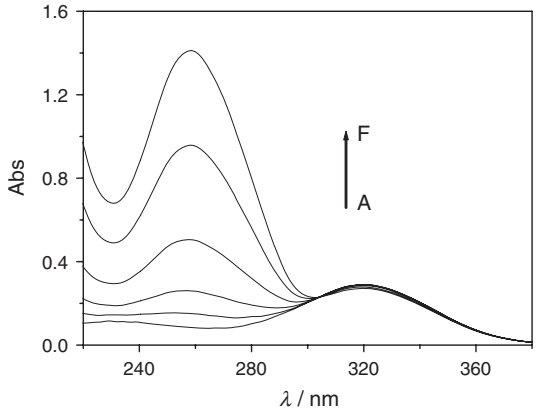

Fig. 6. UV–Vis spectra of 0.3 mM MTZ in BR buffer (pH = 7.0) in the presence of different concentrations of NP: (A) 0; (B) 5.0 μM; (C) 17.2 μM; (D) 45.2 μM; (E) 121.2 μM; (F) 200.8 μM.

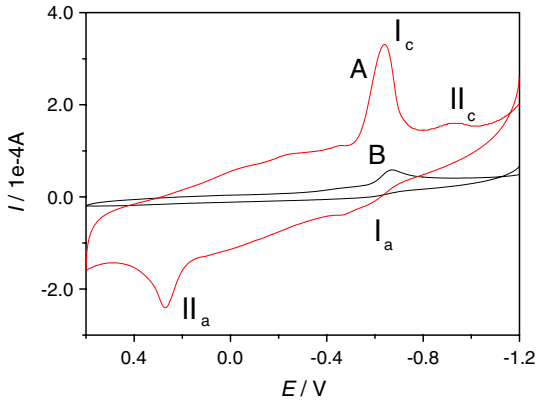

Fig. 7. Cyclic voltammograms of 0.5 mM MTZ in pH 7.0 BR buffer at (A) DNA/GCE and (B) bare GCE. Scan rate = 50 mV/s.

yielded $K = 2.2(\pm 1.3) \times 10^4$ M^{-1} and $s = 0.34$. Obviously, the interaction between MTZ and the solution-phase CTDNA was not as strong as some other aromatic compounds which could intercalate into DNA helix via large conjugated structures.

3.2. Spectroscopic studies

The interaction of MTZ and CTDNA was also characterized by a titration monitored by UV–Vis adsorption. Fig. 6 showed the electronic absorption spectrum of MTZ in pH 7.0 BR buffer and the effect of CTDNA addition. The maximum absorption was at 320 nm for MTZ and 258 nm for DNA. With increasing of DNA concentration, the DNA absorption peak increased and MTZ absorption remained almost constant, which suggests that the interaction of MTZ and DNA is weak and the possibility of intercalative binding between them can be excluded. This was in agreement with the observation by voltammetric titration. However, no specific information can be deduced from the spectroscopic titration.

3.3. Interaction of MTZ with CTDNA immobilized on the GCE surface

We have noted the reports concerning the oxidation of guanine (ca. 1 V vs. SCE), adenine (ca. 1.2 V) and the other bases at even higher potentials. However, our previous study revealed that the Calf thymus DNA can bind covalently on carbon based electrodes under DC potential of 1.8 ± 0.3 V vs. 50 mM NaCl–Ag/AgCl, ca. 1.7 V vs. SCE, forming well-conductive biosensing layers with significantly enlarged effective surface area [33,34]. We have examined the possibility of the oxidation of these based; however, no oxidation waves had been found. Thus, we believe that the redox activity of these bases on DNA is strongly dependent on both the electrode material and the state of DNA. The bases in double-stranded native DNA with long chain should be electrochemically more inactive than those in single-stranded DNA with short chains. On the other hand, if the DNA immobilized covalently on the surface of the carbon electrode, the DNA becomes a part of a huge electroconductive polymeric system, the redox property could be obviously different from a free DNA. By

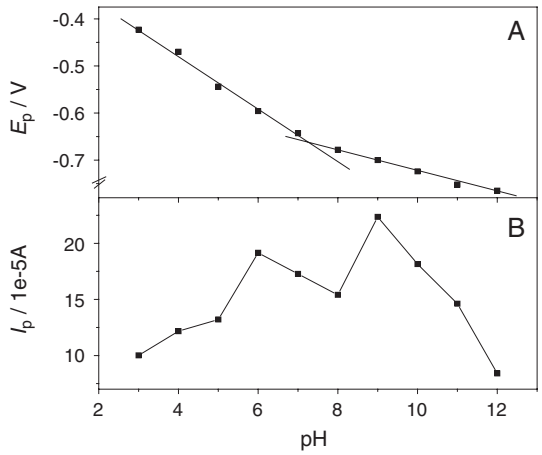

Fig. 8. Effects of pH on the E_p (A) and I_p (B) of 0.2 mM MTZ at DNA/GCE by linear sweep voltammetry. Scan rate = 50 mV/s.

now, our results showed that DNA can be immobilized stably at this potential without oxidation or damage.

Electrochemical behavior of MTZ was investigated by CV at the DNA/GCE and a bare GCE as shown in Fig. 7. Significant differences were visualized. MTZ showed two reduction peaks and two oxidation peaks at DNA/GCE, while only one reduction peak at the bare GCE at −0.662 V which can be attributed to the four-electron/four-proton reduction of nitro group [35,36]. These changes in the cyclic voltammogram can only be due to the interaction of MTZ with the surface-confined DNA. There is obviously a great advantage in using the DNA/GCE to study the interaction mechanism. According to the previous information about the electroreduction of nitroheterocyclic compounds, the two cathodic peaks at −0.623 V and −0.902 V can be ascribed to the splitting of the main peak as depicted by the following reactions [17,18]:

$$R-NO_2 + e^- \leftrightarrows R-NO_2^{\cdot -} \text{(peak Ic and Ia)}$$

$$R-NO_2^{\cdot -} + 3e^- + 4H^+ \rightarrow R-NHOH + H_2O \text{ (peak IIc)}$$

At the DNA/GCE, the reduction of MTZ occurred with a reversible one-electron couple due to the nitro/nitro radical anion redox pair and a second irreversible peak due to the three-electron and four-proton further reduction of the nitro radical anion to the hydroxylamine derivatives. The anodic peak II $_a$ at + 0.271 V may correspond to the electrooxidation of the hydroxylamine derivative generated from II_c:

$$R-NHOH \rightarrow R-NO + 2e^- + 2H^+ \text{(peak IIa)}.$$

In spite of the formation of nitro radical anion at peak I_c and peak I_a was detectable, implying a stabilization effect of the surface-confined DNA on the radical intermediate, the half-life of the radical species was still not high enough to produce a reversible redox couple as for some other nitroimidazoles [37].

The peak current of I_c at the DNA/GCE was nearly 8-fold of the response at the bare GCE and the charging capacity increased 6-fold. It was obviously that the immobilized DNA not only facilitated the electron transfer of MTZ but also enlarged the effective surface area of the electrode. The reduction peak current was proportional to the square root of the scan rate with a slope of 9.31×10^{-4} A $s^{1/2}$ $V^{-1/2}$ from 10 to 100 mV s^{-1} (r = 0.9984), indicating that the reduction process at the DNA/GCE was diffusion controlled. From the slope of the E_p versus log(v) plot, the αn_a value can be evaluated as 0.44. And the diffusion coefficient of MTZ can be calculated as 6.1×10^{-5} cm^2 s^{-1}, one magnitude larger than that at the bare GCE.

The pH effect was examined by linear sweep voltammetry. The result was shown in Fig. 8. Fig. 8A showed two fitting lines obtained as $E_p = -0.2522 - 0.057$ pH (r = 0.9970) from pH 3.0 to pH 7.0, and $E_p = -0.4978 - 0.027$ pH (r = 0.9952) from pH 8.0 to pH 12.0. Since E_p can be expressed as (at 25 °C)

$$E_p = E^0 - (m/n)\ 0.059\,\text{pH} \quad (5)$$

where E^0 is the standard potential, n is the number of electrons transferred and m is the number of protons transferred. Thus m/n of 1 and 0.5 were obtained for the two linear sections, respectively. These results were quite different from the earlier report that no pH dependence was found in alkaline solutions for free MTZ [14,15,25]. It was obviously due to the interactions of reactants and intermediates with the surface-linked DNA. Fig. 8B showed the effects of pH on the peak currents and the maximal value was at pH 9.0. For higher sensitivity, pH 9.0 BR buffer was chosen for the further study.

The obtained differential pulse voltammograms of MTZ at different concentrations in pH 9.0 BR buffer at the DNA/GCE were displayed in Fig. 9. A linearity regression equation of $I_p(A) = 2.01\times10^{-6} + 2.07C(M)$ with r = 0.9955 was obtained. A detection limit was found to be 2.0×10^{-8} M, which is much lower than 10^{-6} M as previously reported [25–27]. A relative standard deviation (R.S.D.) of 4.5% for 5×10^{-6} M MTZ for 5 determinations was obtained. By storing the modified electrode in pH 9.0 BR buffer at 4 °C in a refrigerator, no obvious deterioration on current response was observed for a week and the response declined 10% for a month. These results demonstrated that the DNA/GCE was very sensitive and very good in reproducibility and long-term stability and can be expected for practical applications.

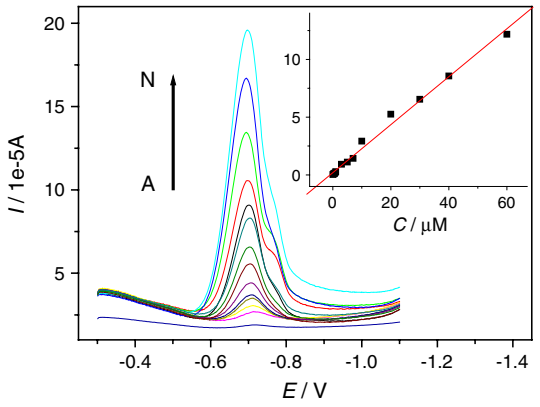

Fig. 9. Differential pulse voltammograms of different concentrations of MTZ in pH 9.0 BR buffer and the corresponding calibration curve (inset) at DNA/GCE, from A to N: 0.05, 0.2, 0.4, 0.6, 0.8, 1.0, 3.0, 5.0, 7.0, 10.0, 20.0, 30.0, 40.0, 60.0 μM. Pulse amplitude = 50 mV, scan rate = 20 mV/s, pulse width = 50 ms.

Table 1
Assay of MTZ in commercial formulations by voltammetric and spectroscopic methods

Formulation	Voltammetric method[a]	R.S.D. (%)	Spectroscopic method[a]	R.S.D. (%)	Relative error (%)	Recovery (%)[a]
Tablet (12020388)	20.8 mg/tablet	3.9	19.7 mg/tablet	2.9	5.6	98.7
Suppository (10940247)	207.4 mg/tablet	4.6	202.6 mg/tablet	3.4	2.4	103.1
Injection (20033600)	2.75 mg/ml	4.2	2.63 mg/ml	2.1	4.6	99.3

R.S.D. = relative standard deviations.
[a] Mean of five experiments.

3.4. Analytical applications

The DNA/GCE was used for determinations of MTZ content in three kinds of commercial formulations, i.e. tablet, suppository and injection. The result obtained is listed in Table 1 in comparison with those determined by spectrometry. A good agreement was found for these two methods with a relative error <6%. Standard addition was used to establish the suitability of the proposed method. Recoveries between 98.7% and 103.1% were obtained, showing high accuracy and repeatability of the proposed voltammetric method.

4. Conclusions

In solution, the drug MTZ can bind to CTDNA with a binding constant of $K = 2.2(\pm 1.3) \times 10^4\ M^{-1}$ and $s = 0.34$ bp, which were successively determined in this work by voltammetric method. Interactions between MTZ and surface-bonded DNA were further studied by using the DNA/GCE. One cathodic peak of MTZ at the bare GCE split into two peaks at the DNA/GCE, which suggested that the reduction of MTZ at the DNA/GCE occurred with a reversible one-electron couple due to the nitro/nitro radical anion redox pair and a second irreversible peak due to the three-electron and four-proton further reduction of the nitro radical anion to the hydroxylamine derivatives. The prepared DNA/GCE was successfully applied in the estimation of MTZ content in commercial dosage forms. It was demonstrated that this electrode as a biosensor is highly sensitive, reproducible and stable. These results are expected to offer new insight into the study of the interaction of drug with DNA.

Acknowledgments

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