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DNA-binding studies of valrubicin as a chemotherapy drug using spectroscopy and electrochemical techniques

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ABSTRACT

In this study, the molecular interactions between valrubicin, an anticancer drug, and fish sperm DNA have been studied in phosphate buffer solution (pH 7.4) using UV–Vis spectrophotometry and cyclic voltammetry techniques. Valrubicin intercalated into double stranded DNA under a weak displacement reaction with methylene blue (MB) molecule in a competitive reaction. The binding constant (k_b) of valrubicin-DNA was determined as 1.75×10^3 L/mol by spectrophotometric titration. The value of non-electrostatic binding constant (k_t^0) was almost constant at different ionic strengths while the ratio of k_t^0/k_b increased from 4.51% to 23.77%. These results indicate that valrubicin binds to ds-DNA via electrostatic and intercalation modes. Thermodynamic parameters including ΔH^0, ΔS^0 and ΔG^0 for valrubicin-DNA interaction were determined as -25.21×10^3 kJ/mol, 1.55×10^2 kJ/mol K and −22.03 kJ/mol, respectively. Cyclic voltammetry study shows a pair of redox peaks for valrubicin at 0.45 V and 0.36 V (vs. Ag/AgCl). The peak currents decreased and peak positions shifted to positive direction in the presence of DNA, showing intercalation mechanism due to the variation in formal potential.

1. Introduction

The study of drug-DNA interactions is very important not only for understanding the mechanism of interactions, but also for the design of new drugs. However, the mechanism of interactions between drug molecules and DNA is still relatively little known. It is necessary to introduce more simple techniques for investigation on the mechanism of interactions for designing new DNA-target drugs and screening in vivo [1,2]. Chemotherapy is an important part of the program for cancer treatment. Some compounds have been developed as potential candidates for anticancer drugs, but only a few of them have been used as effective clinical drugs [3–5]. Many anticancer drugs have been known to interact with DNA to exert their biological activities. Generally, DNA-acting anticancer drugs can be classified into three categories: (i) drugs forming covalent bonding with DNA; (ii) drugs forming non-covalent complexes with DNA by either intercalation or groove-binding; and (iii) those belong to specific drugs with DNA backbone cleavage property [5,6].

Valrubicin (N-trifluoroacetyladriamycin-14-valerate) is a second-generation anthracycline, a cell cytostatic drug derived from the highly effective anthracycline doxorubicin (Adriamycin) [7,8]. Anthracyclines have been known since the 1960s and are accepted as antitumor drugs because of their cytostatic effects [9,10]. Chemotherapeutic drugs are effective molecules; however, they are often associated with severe side effects such as toxicity to skin and tissues [7,11,12]. Since the introduction of valrubicin in 1998, it has been used in bladder cancer therapy [13,14]. The antitumorigenic activity of valrubicin is achieved by contact with the bladder wall and subsequent absorption by cancer cells in which valrubicin expresses its cytostatic effect, resulting in a reduced proliferation of tumoral cells [14,15]. The absence of irritation and skin toxicity as ulceration and necrosis, which is unique among anthracyclines in combination with the negligible systemic absorption on repeated dermal application, makes valrubicin relevant for the treatment of hyperproliferative debilitating but not life-threatening diseases [14–18].

Since the synthesis of carbon nanotubes (CNTs) by Iijima in 1991 [19], CNTs have attracted remarkable attention in chemical, physical and material fields due to their unique structure and extraordinary properties [20]. Due to high electrical conductivity, chemical stability, and extremely high mechanical strength [21], CNTs have been used in different applications as well as sensor fabrication [22,23]. For instance, Hajian et al. [18] immobilized some sequences of oligonu-

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cleotides on gold nanoparticles/CNTs modified glassy carbon electrode for study on the valrubicin-DNA interactions. In this work, the interaction between valrubicin and fish sperm DNA was studied in phosphate buffer solution (pH 7.4) using UV–Vis spectrophotometry and cyclic voltammetry techniques. The aim of the present study was to determine the thermodynamic parameters of interaction (enthalpy, entropy and Gibbs free energy) and suggest the mechanism of molecular interaction with DNA.

2. Experimental

2.1. Reagents and solutions

All chemicals were of analytical grade and used without further purification. Multi-wall carbon nanotubes (MWCNTs) (diameter: 10–20 nm, length: 1–2 μm, purity > 95%) were purchased from Sigma-Aldrich Chemicals (Saint Louis, USA). Valrubicin was purchased from Rockville, USA and used as received. The stock solution of valrubicin (1×10^{-3} mol/L) was prepared by dissolving 0.018 g of valrubicin hydrochloride in ethanol (10 mL) and adjusted to 25 mL with double distilled water. Fish sperm DNA was supplied by Sigma Biological (Saint Louis, USA) and used as received. The stock solution of DNA (1.38×10^{-3} mol/L) was prepared by dissolving 0.010 g of DNA in phosphate buffer (pH 7.4) and completing the volume to 25 mL. The concentration of DNA solution was determined spectrophotometrically using the molar absorptivity (ε_{260}=6600 L/mol cm). Methylene blue solution (1.0×10^{-3} mol/L) was prepared by dissolving 0.0177 g of methylene blue in double-distilled water and diluting to 50 mL.

2.2. Apparatus

Absorption measurements were carried out using double-beam spectrophotometer (Perkin Elmer, Lambda 25, USA) and PG spectrophotometer (T80, UK) equipped with 1.0 cm quartz cells. Voltammetric measurements were carried out using potentiostat electrochemical system (Autolab 302N, Metrohm, Switzerland) connected to a conventional three-electrode system including a modified platinum electrode (PtE/MWCNTs) as working electrode, an Ag/AgCl (3.0 mol/L KCl) electrode as reference electrode and a graphite bare electrode as auxiliary electrode. A pH meter (Metrohm, model 827) was used for adjustment of pH values.

2.3. Analytical procedure

The absorption spectra of valrubicin were recorded in the wavelength range of 200–800 nm. The binding constant (k_b) between double stranded DNA (ds-DNA) and valrubicin was measured by spectrophotometric titration at different temperatures and ionic strengths.

The electrochemical behavior of valrubicin was studied using cyclic voltammetry in the potential range of 0.8–0.0 V (vs. Ag/AgCl).

2.4. Modification of Pt electrode with MWCNTs

The bare Pt electrode was pretreated carefully with 0.05 μm alumina slurry on a polishing cloth, rinsed thoroughly with HNO_3-H_2O (1:1, v/v), and then washed with pure ethanol and double distilled water, respectively. 10 mg of the untreated MWCNTs was added to 25 mL of concentrated nitric acid (wt. 68%) and sonicated for about 4 h. The mixture was filtered and washed with double-distilled water until the filtrate was neutral. The treated MWCNTs were kept in an oven to dry at 50 °C for 2 h. 5.0 mg of the treated MWCNTs was sonicated in 10.0 mL of N,N-dimethylformamide (DMF) for 30 min to prepare a homogeneous suspension. The pretreated PtE was coated evenly with 10.0 μL of MWCNTs suspension and the solvent was evaporated under an ultraviolet lamp. Before using, the modified electrode was washed repeatedly with double distilled water to remove the loosely bound modifier [20].

3. Results and discussion

3.1. Spectrophotometry study on the interaction of valrubicin with ds-DNA

UV–Vis spectroscopy is the most common and convenient technique to study the interaction between small molecules or rare earth complexes with nucleic acids. Molecules containing aromatic or phosphate chromophore groups interact with double helix structure of DNA and the molecular interaction can be studied based on the changes in the absorption spectra [24]. Red shift/blue shift, hyperchromic/hypochromic effects and the isochromatic point are spectral properties for DNA-drug interactions that are closely related to the double helix structure [24,25]. The hypochromicity at the maximum absorbance of DNA (260 nm) indicates the compaction of DNA due to the electrostatic interaction while intercalation mechanism induces the hyperchromicity at this wavelength [24,26].

In order to prove the interaction between valrubicin and ds-DNA, UV–Vis absorption spectra of valrubicin in the presence of different concentrations of DNA were recorded (Fig. 1). In the absence of DNA, valrubicin displayed three absorbance peaks at 260, 300 and 481 nm, respectively. With the addition of DNA, the intensity at 260 nm band increased while the intensity at 481 nm decreased and a partial red shift was observed. This may be attributed to the decrease in the number of chromophores in solution. Based on the variations of absorbance at the absorbance band, the binding constant (k_b) for valrubicin-DNA was determined using Wolfe–Shimer equation [24]:

$$\frac{A_0}{A-A_0}=\frac{\varepsilon_G}{\varepsilon_{H-G}-\varepsilon_G}+\frac{\varepsilon_G}{\varepsilon_{H-G}-\varepsilon_G}\times\frac{1}{k_b[DNA]} \quad (1)$$

In which A_0 and A are the absorbances of valrubicin in the absence and presence of DNA, respectively, and ε_G and $\varepsilon_{H\text{-}G}$ are their respective absorbance coefficients. Plot of $\frac{A_0}{A-A_0}$ versus $\frac{1}{[DNA]}$ was linear (Fig. 2) and the binding constant (k_b) was determined as 1.75×10^3 L/mol from the ratio of the intercept to the slope of linear equation.

3.2. Competitive interaction of valrubicin with MB-DNA complex

In order to better understand the interaction of valrubicin with ds-DNA, methylene blue (MB) was used as a dye probe for competitive interaction with DNA in the presence of valrubicin. As shown in Fig. 3, UV–Vis adsorption spectra of MB in phosphate buffer solution (pH=7.4) have a maximum peak at 662 nm. The absorption intensity declined and slightly shifted to red region (red shifted) in the presence of DNA. The red shift is usually associated with molecular intercalation into the bases of DNA [27]. The strength of the electronic interaction is

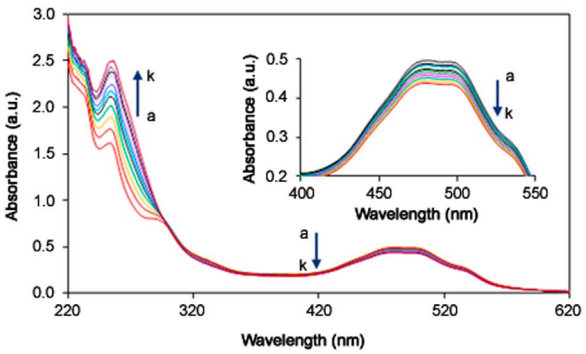

Fig. 1. Absorption spectra of 10 μmol/L valrubicin in the presence of ds-DNA at different concentrations (a: 0.0, b: 7.0, c:14.0, d: 21.0, e: 28.0, f: 35.0, g: 42.0, h: 49.0, i: 56.0, j: 63.0, and k: 70.0 μmol/L in phosphate buffer (0.1 mol/L, pH 7.4)).

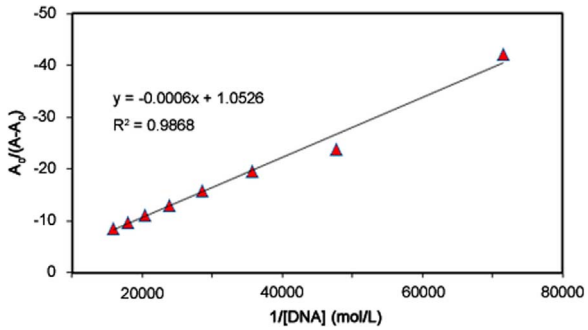

Fig. 2. Plot of $(A_0/A–A_0)$ versus 1/[DNA]. Conditions: $C_{valrubicin}$=10 μmol/L, $C_{phosphate\ buffer}$ =0.1 mol/L, pH =7.4, λ_{max} =481 nm.

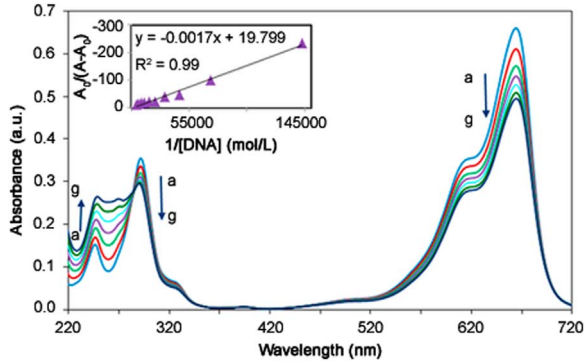

Fig. 3. Absorption spectra of MB in the present of DNA at different concentrations (a: 0.0, b: 5.3, c: 10.0, d: 14.8, e: 19.1, f: 23.0 and g: 26.7 μmol/L, C_{MB}=100.0 μmol/L in phosphate buffer (0.1 mol/L, pH 7.4)). Inset: the binding plot of MB in the presence of DNA at λ_{max}=670 nm.

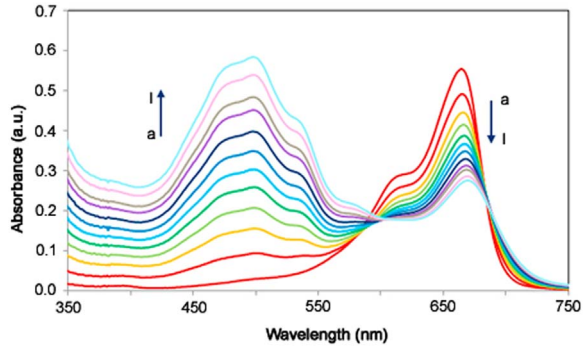

Fig. 4. Absorption spectra of the competitive interaction between valrubicin and MB bonded to ds-DNA. Conditions: $C_{valrubicin}$ = 0.0(a), 10.0(b), 20.0(c), 30.0(d), 40.0(e), 50.0(f), 60.0(g), 70.0(h), 80.0(i), 90.0(j), 100.0(k) and 110(l) μmol/L, C_{MB} = 10.0 μmol/L and C_{DNA} = 50.0 μmol/L in phosphate buffer (0.1 mol/L, pH 7.4).

inversely proportional to the square of the distance between chromophore and DNA bases. Furthermore, the observed spectral changes in the presence of DNA indicate that the distance between intercalated MB and DNA bases is small. Therefore, π electrons of MB combine with π electrons of DNA, and the empty π^* orbital for MB couples with the π orbital of bases, which facilitates a decrease in the energy of π- π^* electron transition and causes red shift [5]. At the same time, the empty π^* orbital is filled by electrons and decreases the energy of the π- π^* level. Similarly, the value of the binding constant for the interaction of MB with DNA was determined by using Eq. (1). As shown in Fig. 3, inset, there is a linear relationship between $\frac{A_0}{A-A_0}$ and $\frac{1}{[DNA]}$, and from this plot, k_b was found to be 1.16×10^4 L/mol. The competitive

Table 1
Hypochromicity (H), total equilibrium binding constant (k_b) and non-electrostatic equilibrium binding constant (k_t^0) and ratio of k_t^0 to k_b at various salt concentrations.

$[K^+]$ (mol/L)	H (%)	k_b (L/mol)	k_t^0 (L/mol)	k_t^0/k_b(%)
0.0001	4.51	1.20×10^4	544.10	4.51
0.001	6.53	7.29×10^3	545.26	7.47
0.005	7.75	4.42×10^3	547.25	12.37
0.010	10.40	2.31×10^3	548.66	23.77

interaction between valrubicin and MB for binding to DNA was studied by successive addition of valrubicin to MB-DNA complex solution and recording UV–Vis spectra in the range of 220–800 nm (Fig. 4). The observed intensity band at 662 nm for DNA-MB complex decreased and shifted to higher wavelengths (red shift) significantly after increasing concentration of valrubicin. The exchange ability of valrubicin with MB is negligible because the binding of MB to DNA is around 10 times stronger and also as it is shown in Fig. 3, MB has a maximum wavelength at 662 nm and is expected to have blue shift during exchange reaction.

3.3. The effect of ionic strength on the binding interaction of valrubicin-DNA

For study on the interaction mode of valrubicin with ds-DNA, the binding constant was determined at different ionic strengths by changing KCl concentration in the range of 0.0001–0.01 mol/L using spectrophotometric titration. As can be seen in Table 1, the binding constant (k_b) for valrubicin depends on the KCl concentration in the solution. It has been reported that the relationship between k_b and salt concentration is nonlinear at higher concentrations [28]. The binding constant decreased by the increase of KCl concentration, indicating that electrostatic interaction is involved in the DNA binding reaction. Non-electrostatic binding constant (k_t^0) at different KCl concentrations was calculated based on polyelectrolyte equation [24,28]:

$$\ln k_b = \ln k_t^0 + Z\xi^{-1}\{\ln(\gamma \pm \delta)\} - Z\Psi(\ln [M^+]) \quad (2)$$

Where k_b is binding constant, Z is partial charge on the binding ligand involved in the DNA interaction as predicted by polyelectrolyte theory, ψ is the fraction of counterions associated with each DNA phosphate (ψ=0.88 for double-stranded B-form DNA), $\gamma_{\pm}$ is the mean activity coefficient for M^+ ion, ζ is $q_p^2/\varepsilon k_b Tb$ (q_p is the charge of a molecule, ε is the bulk dielectric constant and b is the linear charge spacing of the polyelectrolyte in the absence of any associated ions). For DNA in aqueous solution b=1.7 A°, ζ is 4.2 and δ is a constant value (0.56). In Eq. (2), Z is obtained from the slope of the regression line (ln[K^+] versus ln k_b) for the binding of valrubicin to ds-DNA (Fig. 5). Results of the calculations are listed in Table 1 along with hypochromicity (H %) and the percentage of non-electrostatic binding constant (k_t^0) contribution to

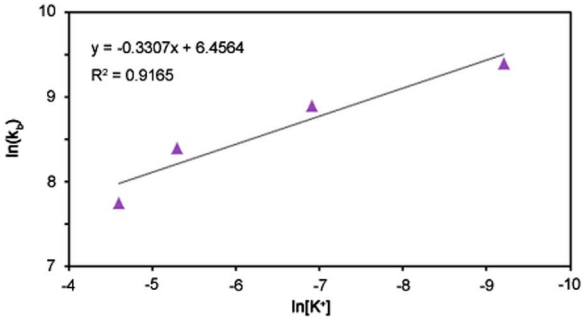

Fig. 5. The effect of $[K^+]$ on the equilibrium binding constant (k_b) for the binding of valrubicin to ds-DNA.

Table 2
The effect of salt concentration (KCl) on free energy changes (ΔG^0,ΔG_{pe}^0 and ΔG_t^0).

$[K^+]$ (mol/L)	ΔG^0 (kJ/mol)	ΔG_{pe}^0 (kJ/mol)	ΔG_t^0 (kJ/mol)	$\Delta G_t^0/\Delta G^0$(%)
0.0001	−23.28	7.546	−30.827	132.413
0.001	−22.04	5.659	−27.697	125.681
0.005	−20.79	4.341	−25.138	120.872
0.010	−19.18	3.773	−22.959	119.666

the total binding constant (k_b) at different KCl concentrations. k_t^0 is a criterion of non-electrostatic forces between molecules and DNA. As can be seen in Table 1, k_b values are salt-dependent but the value of k_t^0 is almost constant at different KCl concentrations. This is consistent with the expectation for the salt-independency of this parameter. Furthermore, whereas the values of k_t^0 were constant throughout the concentrations of salt, the values of k_t^0/k_b increased from 4.51% to 23.77%. These results demonstrate that electrostatic attraction between valrubicin and DNA is governed.

Further analysis was carried out to investigate the standard binding free energy change (ΔG^0) for the binding of valrubicin to ds-DNA into its electrostatic (ΔG_{pe}^0) and non-electrostatic (ΔG_t^0) contributions at a given concentration of KCl. The results of the calculations of energetics for the binding at different KCl concentrations are shown in Table 2. The total binding free energy changes listed in Table 2 were calculated according to the standard Gibbs relation:

$$\Delta G^0 = -RTlnk_b \quad (3)$$

where R and T refer to the gas constant and the temperature in Kelvin, respectively. The salt dependence of the binding constant was determined from the following equation:

$$SK = \delta lnk_b/\delta ln[K^+] = -Z\Psi \quad (4)$$

where SK is equivalent to the number of counterions released upon binding of the molecule with charge Z. The value of Zψ (ψ=0.88) was estimated from the slope of ln[K^+] versus ln k_b (Fig. 5) and Z was calculated as +0.38. The SK value was also used to determine the polyelectrolyte standard free energy change (ΔG_{pe}^0) contribution to the overall free energy change (ΔG^0) at a given KCl concentration by the following equation [24]:

$$\Delta G_{pe}^0 = (SK)RTln[K^+] \quad (5)$$

The difference between Gibbs free energy changes (ΔG^0) and ΔG_{pe}^0 is defined as the non-electrostatic free energy change (ΔG_t^0):

$$\Delta G_t^0 = \Delta G^0 - \Delta G_{pe}^0 \quad (6)$$

As can be seen in Table 2, the standard electrostatic DNA binding free energy change (ΔG_{pe}^0) of valrubicin decreased as the increase of salt concentration as expected by electrolyte theory. In contrast, the non-electrostatic binding free energy change (ΔG_t^0) was almost consistent at different salt concentrations and remained constant.

Table 3
Some thermodynamic parameters for valrubicin binding to ds-DNA at different temperatures.

T (K)	H[a] (%)	k_b (mol/L)	ΔH^0 (kJ/mol)	ΔS^0 (kJ/mol K)
298	4.518	1.20×10^4	-2.52×10^4	1.55×10^2
308	6.539	7.29×10^3		
313	7.755	4.42×10^3		
323	10.402	2.31×10^3		

[a] Hypochromicity.

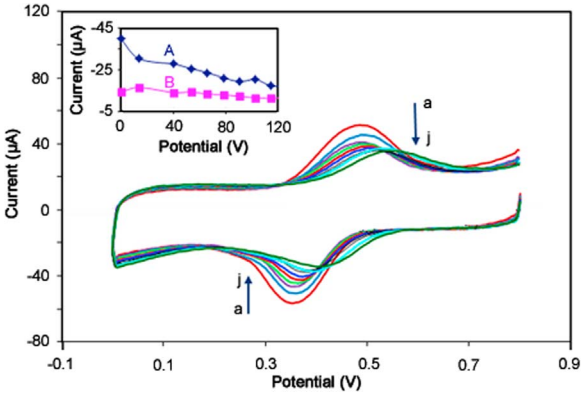

Fig. 6. Cyclic voltammograms of valrubicin on the surface of PtE/MWCNTs in the presence of ds-DNA at different concentrations (a: 0.0, b: 13.77, c: 27.27, d: 40.51, e: 53.5, f: 66.23, g: 78.73, h: 91, i: 103.03, j: 114.85 μmol/L, $C_{valrubicin}$=50 μmol/L in phosphate buffer (0.1 mol/L, pH 7.4) and KCl (0.1 mol/L). Inset: (A) Reduction peak currents of 50 μmol/L valrubicin in the presence of DNA, (B) Cathodic peak currents of $K_3Fe(CN)6$ at 100 μmol/L. Conditions: C_{DNA}=0.0(a), 13.77(b), 40.51(c), 53.5(d), 66.23(e), 78.73(f), 91(g), 103.03(h) and 114.85(i) μmol/L in phosphate buffer (0.1 mol/L, pH 7.4) and KCl (0.1 mol/L).

3.4. Thermodynamic parameters

Study on the thermodynamic parameters of DNA interactions is interesting to understand the driving forces govern the binding of drugs to DNA. In this respect, to investigate the thermodynamic parameters of ds-DNA with valrubicin, the binding constants were calculated at different temperatures by spectrophotometric titration (Table 3). Assuming that the enthalpy change (ΔH^0) is independent of temperature over the range of employed temperatures, ΔH^0 was calculated from the van't Hoff plot (lnk_b vs. 1/T) in the corresponding temperature range [24,29–31]. The striking observation is that valrubicin binds exothermically to ds-DNA because the binding constant for the interaction decreases with temperature.

3.5. Electrochemical behavior of valrubicin in the presence of DNA

Cyclic voltammetric study of valrubicin during successive addition of ds-DNA was conducted on the surface of modified electrode (PtE/MWCNTs) in KCl solution (0.1 mol/L KCl) containing phosphate buffer (0.1 mol/L, pH 7.4). A pair of redox peaks for valrubicin was observed at potentials around 0.36 V and 0.45 V (vs. Ag/AgCl). Upon successive addition of DNA to valrubicin solution, the redox peak currents decreased and shifted to positive values (Fig. 6) due to the formation of valrubicin-DNA adduct with smaller diffusion coefficient. As it is shown in Fig. 6, inset (A), the reduction peak current for $K_3Fe(CN)_6$ has not significantly changed in the presence of DNA, showing no interaction nor blocking of electrode surface while the cathodic peak current for valrubicin decreased sharply upon titration with DNA (Fig. 6 inset (A)). The mode of valrubicin–DNA interaction can be understood from the variation in formal potential in the presence of DNA. In general the positive shift (anodic shift) in formal potential is caused by the intercalation with DNA [32], while negative shift is observed for the electrostatic interaction of a drug with DNA [33]. Therefore, this is evident that positive peak potential shift (anodic shift) in the CV behavior of valrubicin by the addition of DNA is attributed to the intercalation mechanism. The positive shift for oxidation peak shows that valrubicin in reduced form is more stable in the presence of DNA while its oxidation product can be easily reduced on the surface of carbon nanotubes modified electrode after bonding with DNA. Moreover, decrease in peak currents for both peaks is due to the intercalation of valrubicin into DNA.

4. Conclusion

In this work, DNA binding properties of valrubicin, as an anthracycline chemotherapy drug, including binding constant and thermodynamic parameters were studied by spectrophotometry and voltammetry techniques. The molecular interaction caused hypochromicity and red shift in the absorbance spectra of valrubicin in the presence of DNA. Moreover, cyclic voltammetry study shows a remarkable decrease in the redox peak currents upon successive addition of DNA and formal potential shifted to positive direction as a result of intercalation mechanism. Based on the spectrophotometry studies in different ionic strengths, the mode of interaction between valrubicin and ds-DNA is a combination of intercalation and electrostatic due to the planar structure and positive charge of drug molecule.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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