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Adriblastina–single stranded DNA interaction with statistical analysis

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Abstract

The electrochemical behavior of adriblastina has been studied at in situ mercury film electrode (in situ MFE) and platinum electrode (PtE) in the presence of phosphoric acid as supporting electrolyte using Osteryoung square-wave stripping voltammetry (OSWSV) and cyclic voltammetry (CV). Optimal experimental and operational parameters have been selected for the drug accumulation and determination in aqueous medium. The interaction of the drug with single stranded DNA (ssDNA) has been studied and validated by using classical least square and partial least square with propagation of error. The formal potentials E° and $E^{\circ\prime}$ and the equilibrium constants K_1 and K_2 have been calculated. It was found that K_2 for the oxidized form of adriblastina is 63 times than K_1 for the reduced form. Among several possible interfering metal ions, a complex formation reaction was observed between adriblastina and Cu(II) ions at in situ MFE. Cu(II) ions formed 1:2 metal:drug complex which is more stable than ssDNA–drug interaction and consequently it inhibits drug biochemical damage effects. The copper complex offers sub-nanograms determination of adriblastina in that 5.80 and 180 pg/ml could be easily detected in aqueous and urine media, respectively, with R.S.D. less than 4%. *F*-test and *t*-test have been applied in urine media giving good results that indicated the high accuracy of the proposed method.

Keywords: Adriblastina; ssDNA; Copper complex; Urine; Chemometry; Statistical analysis

1. Introduction

Adriblastina is one of the most important anthracycline antibiotics which are among the most important antitumor agents. It is chemically named (8*S*–10*S*)-10-(3-amino-2,3,6-trideoxy-α-L-lyxo-hexopyranosyl) oxy-7,8,9,10-tetrahydro-6,8,11-tri-hydroxy-8-hydroxyacetyl-1-methoxy-5,12-naphthacenedione hydrochloride. The anthracycline antibiotics are currently experiencing wide clinical use in anticancer therapy [1]. They are produced by the fungs strep, peucetius var and caesius. Adriblastina is effective in acute leukemias and malignant lymphomas. It is also active in a number of solid tumors, particularly breast cancer [1]. It is an important ingredient for the successful treatment of ovarian, bladder, Hodgkin's and non Hodgkin's lymphomas, Wilm's tumor and neuroblastoma. The drug is also particularly beneficial in a wide range of sarcomas, including Osteogenic, Edwing's and soft tissue sarcomas. It has demonstrated activity in carcinomas of the endometrium, testes, prostate, cervix, head and neck [1]. In addition, many new analogues and derivatives of adriblastina are being formulated and are coming to clinical trials. The development of sensitive, selective and efficient analytical method for the determination of adriblastina in physiological fluids and tissues is essential for the evaluation and optimized administration of this drug.

A number of important biochemical effects have been described for anthracyclines, any one or all of which could have a role in therapeutic and toxic effects of such drugs. The main biochemical effects are concerned with nucleic acid synthesis. These compounds can intercalate with DNA and this binding is considered responsible for the interference with template DNA function [2]. Scission of DNA is believed to be mediated either by the action of topoisomerase II or by the generation of free radicals [1]. The anthracyclines react with cytochrome reductase in the presence of reduced nicoti-

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namide adenine dinucleotide phosphate (NADPH) to form semiquinone radical intermediates, which in turn can react with oxygen to produce superoxide anion radicals. These can generate both hydrogen peroxide and hydroxyl radicals, which are highly destructive to DNA or RNA. The anthracyclines can also interact with cell membranes and alter their functions; this may play an important part in both the antitumor actions and the cardiac toxicity caused by these drugs. So, adriblastina is considered an important model of anthracyclines for understanding how small molecules interact with DNA in a sequence-specific manner. In addition, metal ions are present in all biological processes involving the nucleic acids; the occurrence of metal chelates inside the cell may be an important step in the course of inhibition DNA damage. Therefore, the complexation of adriblastina by metal ions has been studied to give a route to modify the aglycon moiety, and to get new compounds, which may exhibit less toxicity.

The reported methods of analysis for the drug include flourimetry [3,4], radio-immunossay [5,6], thin layer chromatography [7,8] and high performance liquid chromatography [9–14]. In addition there are few methods for the analysis of drug using UV–vis spectrophotometry [15–17]. Adriblastina was determined by ac polarography [18], differential-pulse polarography [19] and adsorptive stripping voltammetry [20]. There are few procedures for the voltammetric determination of the drug on solid electrodes [21,22]. These methods utilized adsorption of the drug onto a carbon paste electrode with flow injection approach [21] and wax-impregnated graphite electrode [22]. Adriblastina was also used as intercalator for the determination of double stranded DNA on a hanging mercury drop electrode [23] and its in situ interaction with double helix DNA was recently investigated using a voltammetric DNA-biosensor [24,25].

The aim of the present work is to give a scope on the electrochemical behavior, determination and interaction of the drug with ssDNA at two solid electrodes: in situ mercury film electrode and platinum electrode. Furthermore, the drug–ssDNA interaction has been investigated and validated by using classical least square and partial least square with propagation of error. Also, the possible complexation reaction between Cu(II) ions and adriblastina and its effect on the drug–DNA interaction has been reported. The method was applied for the determination of drug in urine samples.

2. Experimental

2.1. Reagents and solutions

A fresh solution of adriblastina was prepared daily in doubly distilled water. The solutions are diluted as required for standard additions. Adriblastina was provided by Pharmacia and Upjohn S.P.A., Milan, Italy. It was used without any further purification. Different types of supporting electrolytes were used, e.g. phosphoric acid, acetate, borate, Britton–Robinson, isotonic Sorensen and HEPES buffers. Phosphoric acid (0.027 M) in the presence of required volume of free-carbonate sodium hydroxide—to desire the required pH value—was chosen as supporting electrolyte that gives the best signal. Stock solution of mercuric ion (10^{-2} mol/l) is prepared by dissolving the required weight of basic mercuric nitrate (May & Baker Ltd., Dagenham, England) in doubly distilled water. Single stranded calf thymus DNA for molecular biology was of Sigma quality (Lot 43H67951). It was prepared (5 mg in 5 ml injection water) by a modification of the method of Alberts and Herrick using calf thymus DNA, D 1501. The resulting solution was divided into 10 aliquots (for daily use) and was kept in polyethylene vials frozen. Urine samples were taken from patients undergoing adriblastina chemotherapy. The preparation of urine samples for the drug determination is made as mentioned before [26]. All other reagents were of grade quality.

Solutions of diverse ions used for interference studies were prepared using the nitrate, sulphate or perchlorate salts of metal ions. Dilute acids were some times added to prevent the hydrolysis of metal ions whenever needed.

2.2. Instrumentation

Voltammetric measurements are recorded using the CV-50W Voltammetric Analyzer (USA) electrochemical running under windows^{TM} software. All controlled parameters are entered through a BAS/windows interface. This information is transferred to the CV-50W microprocessor where optimum hardware settings are calculated for the specified technique. These values are loaded automatically and upon applying the command run, data are collected and transmitted to the PC where it is displayed in virtual real time. Standard C-2 cell stand is fully shielded in a Faraday cage (EF-1080) with three electrodes: a glassy carbon (MF-2012, diameter 3 mm), or platinum electrode (MF-2013, diameter 50 μm) working electrode, a silver/silver chloride reference electrode (MF-2063) and a platinum wire auxiliary electrode (MW-1032). Voltammograms are collected using hp HEWLETT PACKARD laser jet 4L printer. These data were sometimes plotted using Microsoft Excel program utilizing either the line or scatter presentation.

The pHs were justified by using the Fisher Scientific Accument pH Meter Model 810 equipped with a combined glass electrode, which is calibrated regularly with buffer solutions (pH 4.00 and 7.00) at 25 ± 1 °C. V3 series HTL micropipettes (Germany) were used to pipette μl volumes of solutions.

2.3. Recommended procedures

A 10 ml volume containing 0.027 M of phosphoric acid in the presence of required volume of free-carbonate sodium hydroxide to the desired pH value is added as supporting electrolyte to cell and degassed with highly purified nitrogen for 8 min. The in situ mercury film electrode (in situ MFE) was prepared by adding a definite volume of basic mercuric

nitrate solution directly to the sample solution and simultaneously depositing mercury ions and the drug. Drug molecules are then stripped from the mercury thin film by scanning Osteryoung square-wave potential. This electrode combines the sensitivity of thin films with high selectivity and reproducibility. The voltammogram is recorded after definite quiet time by applying Osteryoung square-wave potential scan from −900 to −100 mV at pH 5.9. MFE electrode surface is polished by simply wiping with BAS cloth disk coated with two drops of alumina (CF-1050) and doubly distilled water. The voltammograms were also recorded by applying Osteryoung square-wave potential scan from +1400 to −1100 mV on the surface of Pt electrode at pH 6.8. Its surface is polished after each run by using BAS fine diamond polish. The solution is stirred by using stirring magnet (ER-9132) at constant stirring rate (100 rpm). The interaction of ssDNA with adriblastina is studied by titration of different ssDNA concentrations with a fixed concentration of the drug and vice versa. The effect of the possible interfering metal ions was studied; the complexation of Cu(II) ions with adriblastina and its effect on drug–ssDNA interaction has been studied at in situ MFE. All results were obtained at room temperature (25 ± 2 °C).

3. Results and discussion

Preliminary investigations of the electrochemical behaviour of adriblastina at various types of solid working electrodes—carbon paste electrode (CPE), glassy carbon electrode (GCE), platinum electrode (PtE) and in situ mercury film electrode—were done. Osteryoung square-wave stripping voltammograms of 1×10^{-5} M adriblastina after 60 s accumulation time at pH 7.4 have been recorded. The best signals with good shape have been appeared at in situ mercury film electrode and platinum electrode. Therefore, these electrodes have been chosen for further investigations. Another reason is that there are no data in the literature dealing with investigation of the drug at these electrodes whereas there are some data at the GCE and CPE's.

3.1. Electrochemical behavior of adriblastina

Voltammetric behavior of adriblastina in solutions at different pH values was carried out in 0.027 M phosphoric acid at in situ MFE and PtE. OSWV mode with high scan rates was used to enhance the adriblastina adsorption current relative to the diffusion controlled one. The square-wave voltammograms of 1×10^{-6} M adriblastina in the pH range 1.7–8.0 at in situ MFE show one reduction peak and three oxidation peaks. Fig. 1A indicates the effect of pH values on the reduction peak of adriblastina; the peak potential is highly dependent on the pH. These results indicate the involvement of proton consumption during the electrochemical process. The cathodic peak of adriblastina is due to the reduction of anthracycline's quinone center to hydroquinone. In a related analytical study, Sternson and Thomas [19] described the use of differential pulse polarography for the determination of the drug in blood plasma giving data similar to our suggested one.

The hydrodynamic plot (Fig. 1B) indicates the effect of pH on adriblastina oxidation peaks. The first oxidation peak (I) is located at about −0.52 V in acidic media and shifted to −0.68 V in the pH range 4.0–6.9; then it disappeared

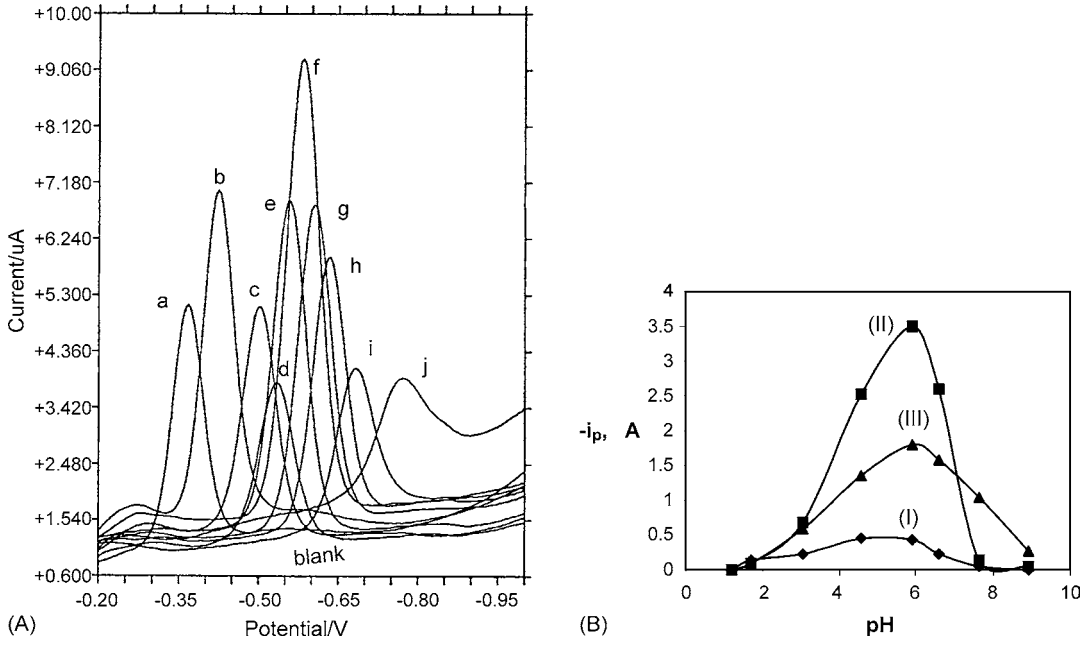


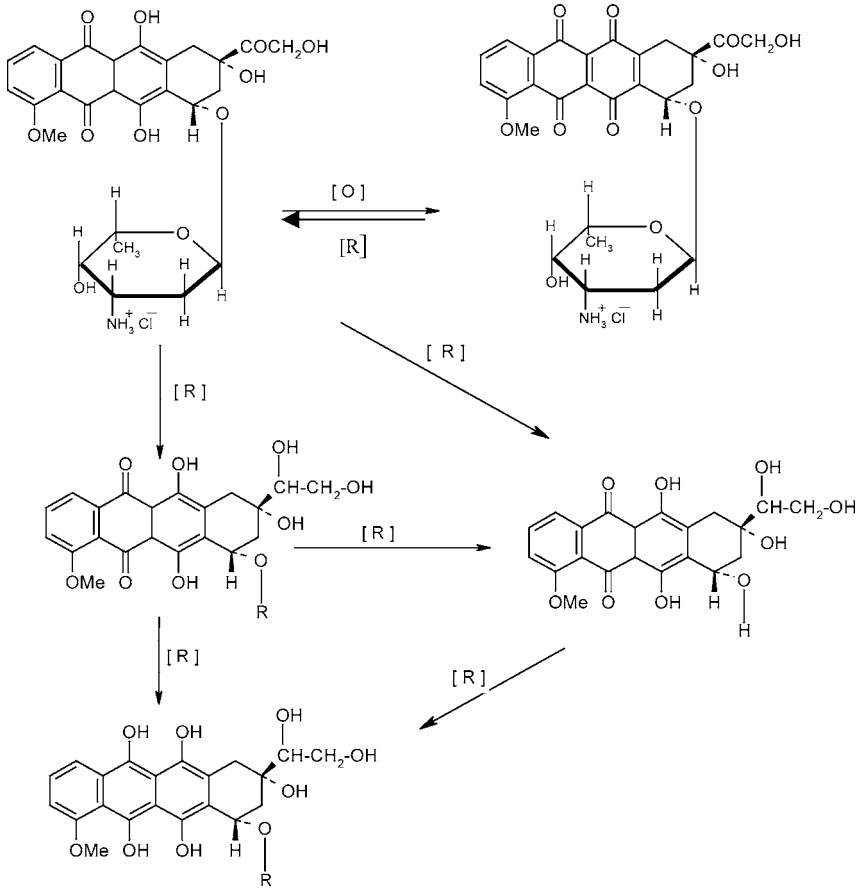
Fig. 1. (A) Effect of pH on cathodic square-wave stripping voltammograms of 1×10^{-6} M adriblastina at in situ MFE in 0.027 M H_3PO_4; pHs: (a) 1.72, (b) 2.34, (c) 3.41, (d) 3.89, (e) 4.33, (f) 4.87, (g) 5.33, (h) 5.82, (i) 6.79 and (j) 7.95. (B) Effect of pH on i_p of oxidation peaks (I–III) of 1×10^{-6} M adriblastina at in situ MFE. Other conditions as cited in (A).

completely in alkaline media, probably due to the drug hydrolysis. Shifting of peak (I) potential (0.2 V/pH unit) is due to the deprotonation equilibrium step. As follows, there is an independency of the peak potential on the pH in neutral media supporting the possibility of none proton consumption; thus, peak (I) may be attributed to the adsorption character of adriblastina at MFE surface in the pH range (4–7). The second oxidation peak (II) is more pronounced than the first one; it appeared at about −0.48 V (versus Ag/AgCl), pH 5.9 and it consisted of an apparently reversible anodic and cathodic pair. Peak (II) potential is highly dependent on pH values indicating the involvement of proton consumption during the electrochemical process. Therefore, the appearance of peak (II) is due to the reoxidation of carbonyl groups at C(5) and C(12) in quinone center of adriblastina. The best sensitivity is achieved at pH 5.9 and then decreased gradually until it is completely disappeared after pH 9.0, probably due to the complete drug hydrolysis. The third oxidation peak (III) is located at about −0.08 V, pH 5.9 and shifted to more electronegative potentials with increasing pH values. Therefore, the electrochemical behavior of peak (III) is similar to that of peak (II) and can be attributed to the oxidation of hydroxyl groups at C(6) and C(11) in the hydroquinone center of the drug.

The electrochemical behavior of adriblastina (1×10^{-6} M) at PtE surface was also studied in the presence of 0.027 M phosphoric acid and required volume of free-carbonate sodium hydroxide. It was found that there are two reduction peaks and one oxidation peak. The first reduction peak is located at about +0.05 V, pH 5.0 and shifted to more electronegative potentials with increasing pHs. This peak shows completely irreversible manner and may be attributed to the reductive cleavage of the daunosamine moiety with the formation of 10-deoxyadriamycinone [27]. The second reduction peak potential (−0.75 V at pH 5.0) is highly dependent on the pHs and it consists of apparently reversible cathodic and anodic pair; therefore, it could be attributed to the redox process for carbonyl groups in the quinone center of the drug. The shape of this peak is worse due to its closely located to the hydrogen evolution potential. Therefore, the first reduction peak has been chosen for further studies at PtE. The suggested electrochemical behavior of adriblastina which is similar to the metabolic transformation of the drug in laboratory animals and in man [28] can be illustrated by Scheme 1.

3.2. Cyclic voltammetric measurements

The nature of the electrochemical process has been carried out applying cyclic voltammetric technique at different pH values. Fig. 2 shows the effect of pH on cyclic voltammo-



Scheme 1.

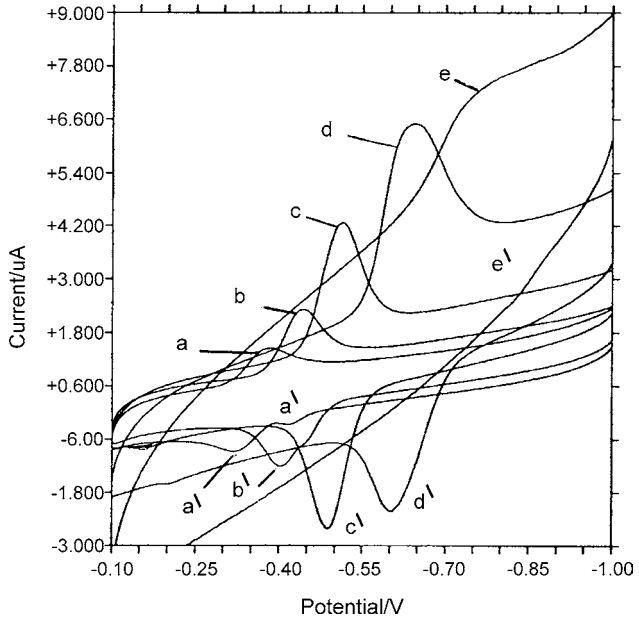


Fig. 2. Effect of pH on the cyclic voltammograms of 1×10^{-5} M adriblastina at in situ MFE applying scan rate 200 mV/s; pHs: (a) 1.86, (b) 3.7, (c) 5.9, (d) 7.8 and (e) 8.5.

grams of adriblastina at in situ MFE surface with scan rate of 200 mV/s. It was found that one reduction peak at about −0.51 V (peak c) and one oxidation peak at about −0.48 V is obtained at pH 5.9 (peak c′) validating the presence of $2H^+/2e^-$ redox process in neutral media. These peaks completely disappeared above pH 8.5. There is another oxidation peak at about −0.33 V (peak a′) in highly acidic media and then disappeared in neutral and alkaline media. The effect of scan rate has been tested on 4×10^{-6} M adriblastina, pH 5.9. Linear relationship is obtained between scan rate and peak height with slope tends to unity (0.96 μA s V^{-1}) and correlation coefficient (0.9991) indicating an adsorption behavior of the drug. The peaks also decreased rapidly upon repetitive scans with a little shift in potentials, indicating fast desorption from the electrode surface. Therefore, rapid scan rates are required for such measurements. The cyclic voltammetric studies of adriblastina have been also evaluated at PtE giving similar adsorption characters of the drug to that at MFE.

3.3. Analytical aspects at the MFE and PtE

The effect of different experimental parameters, e.g. wave form, amplitude, frequency, stirring rate, step potential . . . has been tested at the MFE and PtE. Between three different techniques, viz. linear sweep stripping voltammetry (LSSV), OSWSV and differential pulse stripping voltammetry (DPSV), OSWSV is found to be much sensitive than either LSSV or DPSV with about 14 times increment at MFE and about 2 times at PtE. The effect of mercury film thickness (mercuric ion concentration) on the determination of adriblastina has been tested. It was found that the best sensitivity and reproducibility of the drug measurement is achieved in the presence of 1×10^{-4} M Hg giving thickness of 2 μm at GCE surface. The thickness of mercury film disk electrode (ℓ) was calculated from the following equation [29]:

$$\ell = 2.43\frac{it}{r^2}$$

where i is the mercury ion deposition current, t the deposition time and r is the disk radius.

Under the foregoing optimal parameters, the adsorptive preconcentration of adriblastina and the application of a subsequent square-wave scan in the positive direction gave rise to redox process in phosphoric acid. A large response after 60 s of accumulation time for an assay concentration of 5×10^{-8} M adriblastina is obtained greater than the direct response ($t = 0$ s) indicating the rapid adsorption of adriblastina molecules. The effect of accumulation times ($t_{acc.}$) on the anodic stripping peak current of 5×10^{-9} M (2.9 ng/ml) adriblastina at pH 5.9 onto in situ MFE has been investigated in Fig. 3A. The linearity limits of time at different concentrations of adriblastina are studied and it was found that the linear relationships are obtained up to 300 s in the presence of 0.05 μg/ml adriblastina and decreased to be 120 s in the presence of 5.8 μg/ml. The effect of accumulation time on the cathodic peak of drug (2×10^{-8} M, 11 ng/ml) was also studied onto PtE at pH 6.8 (Fig. 3B). It was found that the accumulation potential is shifted to more negative value by 50 mV with increasing accumulation time after 120 s. This may be due to the reorientation of drug molecules at the electrode surface.

The stability of adriblastina in aqueous solutions has been determined by AdASV and AdCSV onto MFE and PtE, respectively. It was found that adriblastina is stable for about 3 days in weakly acidic and neutral media but decomposes rapidly as pH increased from 8.5 to 12.

The charge of the double layer (Q_{dl}) is calculated by applying a double potential-step chronocoulometric technique for the supporting electrolyte and then for 8×10^{-7} M adriblastina. The adsorbed charge values (Q_{ads}) have been calculated and found to be 6.5 and 3.8 μC at MFE and PtE, respectively. The surface coverage can be measured from the division of the number of Coulombs transferred by the conversion quantity (nFA) yielding coverage of 6.98×10^{-11} and 5.09×10^{-11} mol cm^{-2} at MFE and PtE, respectively. Thus, every one mole adriblastina occupies an area of 2.764 nm^2 at MFE and 1.115 nm^2 at PtE.

3.4. Quantitative utility

Voltammograms of increasing adriblastina concentrations resulted in calibration plots (Fig. 4) with anodic potential scan on MFE at pH 5.9 (A) and with cathodic potential scan on PtE at pH 6.8 (B) applying 0 s (a), 30 s (b), 60 s (c), 120 s (d) and 240 s (e) of preconcentration. The stripping peak currents increase linearly with increasing their concentrations up to 1 μM at lower accumulation times (curves b and c) and up to 0.73 μM at higher ones (curves d and e). Other slopes

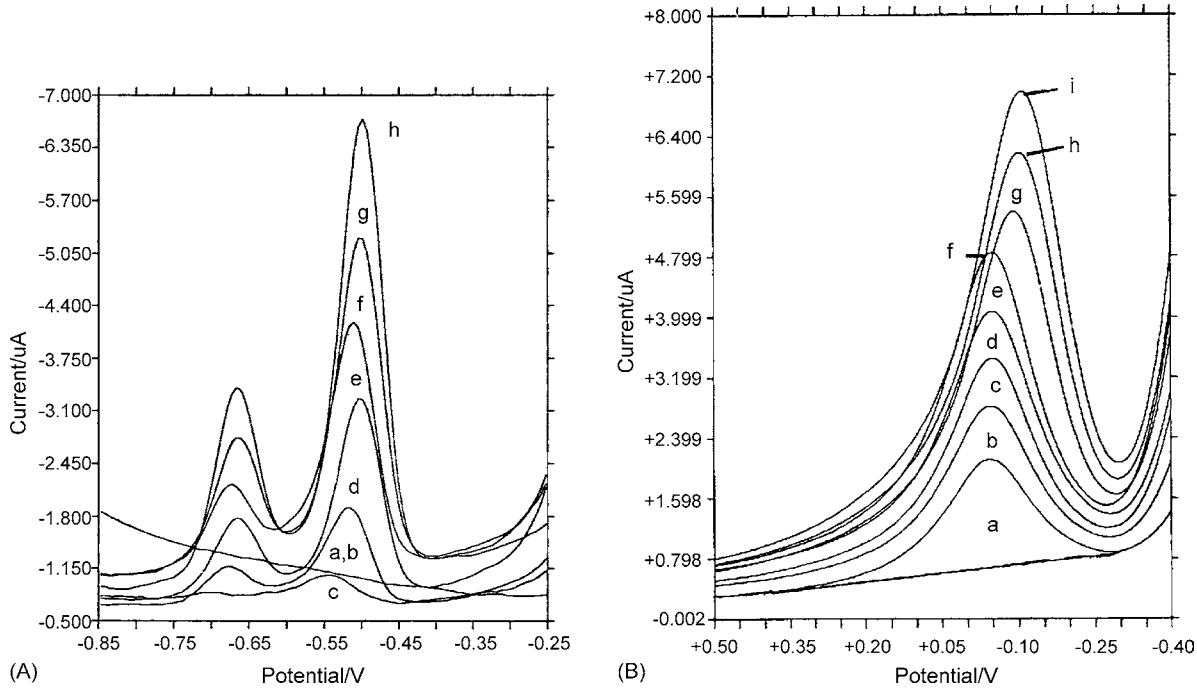


Fig. 3. The effect of accumulation times ($t_{acc.}$) in the presence of 0.027 M H_3PO_4 on the anodic stripping peak current of 2.9 ng/ml adriblastina at pH 5.9 onto in situ MFE (A) and the cathodic stripping peak current of 0.011 μg/ml adriblastina at pH 6.8 onto PtE (B) under the optimal conditions as cited in the text: (a) blank, (b) 0 s, (c) 30 s, (d) 60 s, (e) 120 s, (f) 180 s, (g) 240 s, (h) 300 s and (i) 360 s.

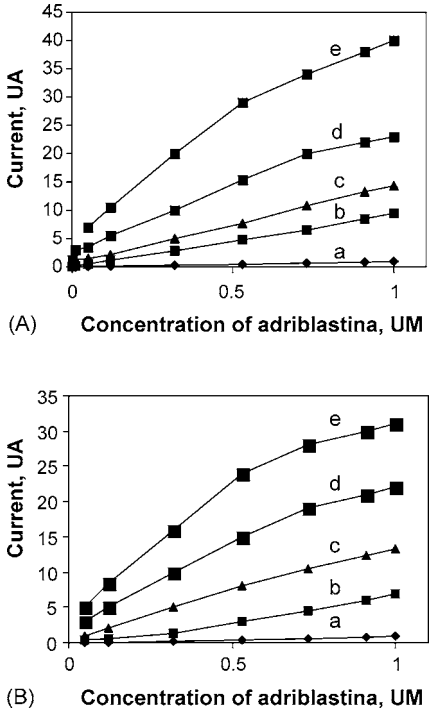


Fig. 4. Calibration plots of different adriblastina spikes from 1 nM to 1.0 μM on in situ MFE with anodic potential scan at pH 5.9 (A) and with cathodic potential scan at pH 6.8 on PtE (B) applying 0 s (a), 30 s (b), 60 s (c), 120 s (d) and 240 s (e) of preconcentration. Other parameters as cited in the text.

Table 1
Characteristic features of the calibration graphs for adriblastina at in situ MFE (A) and PtE (B) after 60 s accumulation

	ASV (A)	CSV (B)
Concentration range (M)	5×10^{-10} to 1×10^{-6}	1×10^{-9} to 1×10^{-6}
Equation (μA μM^{-1})	$Y = 0.92X + 2.0$	$Y = 0.79X + 0.8$
Limit of linearity (M)	1×10^{-6}	1×10^{-6}
Relative standard deviation	1.8623	1.9113
Correlation coefficient	0.9989	0.9978

are obtained in higher concentration ranges at 120 (curve d) and 240 (curve e) accumulation times probably due to reorientation of the drug molecules adsorbed on the surface of MFE and PtE. The calculations of these calibration plots after 60 s accumulation time are cited in Table 1.

The adsorptive accumulations of adriblastina resulted with high reproducible stripping peak currents. For 10 successive measurements of 0.029 μg/ml adriblastina (5×10^{-8} M) applying 60 s accumulation on MFE and PtE, the mean peak current was 0.61 and 0.51 μA with standard deviations 0.062 and 0.059, respectively. A detection limit of 0.116 ng/ml adriblastina (2×10^{-10} M) is easily achieved using the foregoing optimal conditions applying 240 s accumulation. Also, the detection limit (dL) was theoretically calculated using the equation dL = 3S.D./a [30] where S.D. is the standard deviation and a is the slope of the calibration curve. It was found to be 0.049 ng/ml adriblastina (8.5×10^{-11} M). This dL value is more sensitive than those resulted in other publications [21,22] indicating the high sensitivity of our procedure.

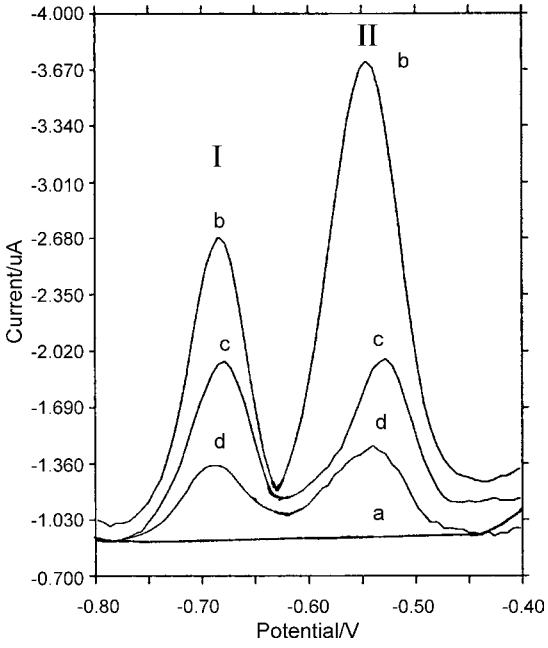


Fig. 5. The OSWS voltammograms at in situ MFE of 0.027 M phosphoric acid, pH 5.9 as supporting electrolyte (peak a), (peak b) $a + 0.25$ ng/ml adriblastina, (peak c) $b + 1$ μg/ml ssDNA and (peak d) $b + 2$ μg/ml ssDNA under conditions as cited in the text.

3.5. Electrochemical studies of adriblastina–ssDNA interaction

The electrochemical investigation of the possible interaction of adriblastina with ssDNA in phosphoric acid onto in situ MFE and PtE under the foregoing experimental conditions has been studied. The interaction varied with the nature of supporting electrolyte, ionic strength, pH value and temperature [31,32]. Strongly acidic and alkaline media have been avoided to prevent any denaturing of DNA molecules. Fig. 5 shows the OSWS voltammograms at in situ MFE, pH 5.9 of supporting electrolyte (peak a) and in the presence of 0.25 ng/ml adriblastina (peak b). The effect of spiked ssDNA on the adsorptive anodic square-wave stripping voltammogram of adriblastina is indicated by peaks (c and d) after 60 s accumulation time. It is obvious that the oxidation peak (I) height is decreased by adding ssDNA molecules; this depression may be due to only the adsorptive competition between ssDNA molecules and drug molecules at the electrode surface. There is also a one-third depression in the height of peak (II) in the presence of 1.0 μg/ml ssDNA with shifting in potential (about 45 mV to more positive potential). This depression is due to the decrease in the number of adsorbed adriblastina molecules on the electrode surface when interacted with ssDNA molecules. As it is well-known that the interactions between the anthracycline moieties and the DNA bases may be due to hydrogen bonding between certain groups on the drug molecules and on the DNA bases or electrostatic attractions [1]. Therefore, we postulated that the mechanism of adriblastina–ssDNA interaction is that the formation of intermolecular hydrogen bond between –C=O group in the oxidized form of the drug and $-NH_2$ group in guanine moiety. Another explanation is that the DNA–drug interaction could be attributed to the electrostatic interaction between the protonated amine group of sugar residue of adriblastina and the negatively charged phosphate group of DNA is far out under our conditions in which the predominant form of the drug is the neutral one. The interaction of ssDNA with drug was also studied by applying adsorptive cathodic square-wave voltammetry. It is observed that there is a little decrease in peak current of the drug (Table 2) with no shifting in potential. Therefore, ssDNA is reacted strongly with the oxidized form of the drug compared to that with the reduced form.

Table 2
The depression values in anodic and cathodic peak currents of 0.069 μg/ml adriblastina onto in situ MFE (A) and PtE (B) with adding ssDNA concentrations

Concentration (μg/ml)	ASV (A) (μA)	CSV (B) (μA)
In absence of ssDNA	2.17	1.91
In the presence of 0.1	2.05	1.89
In the presence of 0.5	1.36	1.82
In the presence of 1.0	0.69	1.76
In the presence of 2.0	0.39	1.71

Cyclic voltammetry was also used to prove our experimental observations. The formal potentials [33] for adriblastina in the absence of ssDNA (E°) and in the presence of ssDNA ($E^{\circ\prime}$) with scan rate 200 mV/s have been calculated. It was found that there is a positive shift in formal potential (about 53 mV); we could then calculate the ratio of the equilibrium constant (K_1) between the reduced free form and the reduced bounded form and the equilibrium constant (K_2) between the oxidized free form and the oxidized bounded form by applying the following equation [33]:

$$2(E^{\circ\prime} - E^\circ) = 0.059 \log\left(\frac{K_2}{K_1}\right)$$

It was found that K_2 is 63 times more than K_1. This means that ssDNA is highly interacted with the oxidized form, which contains carbonyl group as well as the possibility of the presence of electrostataic repulsion between negatively charged ssDNA phophate backbone and the reduced adriblastina. Therefore, it is concluded that the carbonyl group— in the oxidized form—is the predominant functional group for the interaction between ssDNA and adriblastina. The effect of scan rate on the cyclic voltammograms of drug–ssDNA interaction was studied. The relationship between scan rate and the peak height gave a linear curve with slope values 0.91 and 0.93 $\mu A\,s\,V^{-1}$ and correlation coefficient of 0.9983 and 0.9912 at in situ MFE and PtE, respectively, indicating an adsorption behavior of adriblastina–ssDNA complex.

Classical least square and partial least square with propagation of error have been also used for investigation of adriblastina–ssDNA interaction at MFE and PtE. The experimental data which obtained by the standard addition of

adriblastina in the absence and presence of ssDNA have been fitted with calculating the uncertainties in the current (Y-axis), slope (b) and intercept (a) values.

Least square equation of adriblastina in the absence of ssDNA after 60 s accumulations under the optimal conditions at in situ MFE was found to be $Y \pm 0.312 = (14.013 \pm 0.278)X + (0.442 \pm 0.154)$ with correlation coefficient ($r = 0.9981$) entire the linearity range 0.58 ng/ml–0.58 μg/ml of adriblastina. Meanwhile, linear fit equation in the presence of constant ssDNA concentration (0.5 μg/ml) was found to be $Y \pm 0.151 = (13.325 \pm 0.701)X - (0.121 \pm 0.020)$ with correlation coefficient ($r = 0.9994$). The depression in the slope value of fitted equations by introducing ssDNA molecules proves the interaction between adriblastina and ssDNA.

The linear least square equations for the standard addition of drug onto PtE have been also calculated giving the following data:

$$Y \pm 0.000 = 14.142 \pm 0.000X + 0.348 \pm 0.000 \text{ (in the absence of ssDNA)}$$

with correlation coefficient ($r = 1.000$) entire the linearity range from 0.029 μg/ml (5×10^{-8} M) to 0.44 μg/ml (7.5×10^{-7} M) of adriblastina, and

$$Y \pm 0.000 = 14.138 \pm 0.000X + 0.251 \pm 0.000 \times \text{(in the presence of 0.5 mg/ml ssDNA)}$$

with correlation coefficient ($r = 1.000$).

From the above results, it is obvious that the difference in slopes (Δb) between fitted equations in the presence and absence of ssDNA at MFE ($\Delta b = b_{\text{without DNA}} - b_{\text{with DNA}} = 0.688$) is higher than that at PtE ($\Delta b = 0.004$) indicating the important role of the electrode substrate for investigating the interaction between adriblastina and ssDNA molecules. Therefore, the in situ MFE is more pronouncing for investigation of drug–ssDNA complex than PtE.

The method of least square has been also used for designing a model for the indirect ultra-trace determination of ssDNA after 60 s accumulation in the presence of fixed concentration 0.069 μg/ml of adriblastina at MFE and PtE by applying the following equations:

$$Y \pm 0.061 = (-1.504 \pm 0.096)X + (2.168 \pm 0.061) \text{ (at MFE)}$$

with correlation coefficient ($r = 0.995$) entire the linearity range 0.1–1.0 μg/ml ssDNA.

$$Y \pm 0.000 = -0.090 \pm 0.000X + (1.876 \pm 0.000) \text{ (at PtE)}$$

with correlation coefficient ($r = 0.987$) entire the linearity range 0.1–2.0 μg/ml ssDNA.

It was also observed that the calculated standard deviations for Y, a and b at PtE are so small comparing to these at MFE indicating the high reproducibility of the adsorption process at PtE surface. This could be attributed to the re-dissolving of mercury film with longer accumulation times; therefore, the measurement at MFE is preferable with short analysis times.

3.6. Effect of the possible interfering metal ions

A growing body of evidence suggests that the pharmacological activity [34,35] of adriblastina is related to metal ions. As metal ions are present in all biological processes involving nucleic acids, the occurrence of metal chelates with adriblastina inside the cell may be an important step in the course of inhibition for its DNA damage. On the other hand, the cardiotoxicity of the drug has prompted us to search for a noncardiotoxic yet active anthracycline antibiotic. The complexation of adriblastina by metal ions appears to be a route to modify the aglycon moiety, and to get new compounds that may exhibit less toxicity. Therefore, it is very important to investigate the electrochemical characteristic of the metal–adriblastina complexes [36–39].

Among several possible interfering metal ions viz., Cu(II), Fe(III), Fe(II), Zn(II), Ca(II), Ni(II), . . ., a complex formation reaction was observed between adriblastina and Cu(II) ions at in situ MFE. Cyclic voltammetry and Osteryoung square-wave stripping voltammetry were used for such study.

3.6.1. Voltammetric study of Cu(II) complex with adriblastina and its effect on drug–ssDNA interaction

Preliminary tests for adriblastina–Cu(II) complex with an interfacial accumulation behavior at in situ MFE were done. Effect of nature and concentration of supporting electrolyte and pH of the solution has strongly affected electrochemical behavior of the complex, its sensitivity and its selectivity. Best signals were obtained in the presence of HEPES buffer at pH 7.4. Fig. 6A shows a comparison between the OSWS voltammograms of supporting electrolyte (peak a), 2.5×10^{-8} M Cu(II) (peak b), 5×10^{-8} M (0.029 μg/ml) adriblastina in the absence of Cu(II) ions (peak c) and in the presence of Cu(II) ions (peak d) at in situ MFE. The increment in the signal of Cu–adriblastina peak is about 2.5 times adriblastina alone, with a little shift in potential (about 31 mV to more positive value). This indicates the formation of Cu–adriblastina complex with adsorbable behavior.

Effect of Cu(II) concentrations on the sensitivity of its complex with adriblastina has been studied. The best sensitivity was achieved in the presence of 1:2 Cu(II):adriblastina concentration ratio elucidating the 1:2 metal:drug stoichiometry for the formed complex.

Effect of operating parameters, e.g. wave form, pulse amplitude, frequency, step E, stirring rate, . . . on the complex peak response has been investigated using three different stripping wave forms (LSSV, OSWSV and DPSV) of 3×10^{-8} M adriblastina in the presence of 1.5×10^{-8} M Cu(II) after 120 s accumulation. OSWSV shows a peak current value of 4.6 μA at about −0.32 V, which is more sensitive than LSSV (0.8 μA at −0.31 V) and DPSV (0.4 μA at

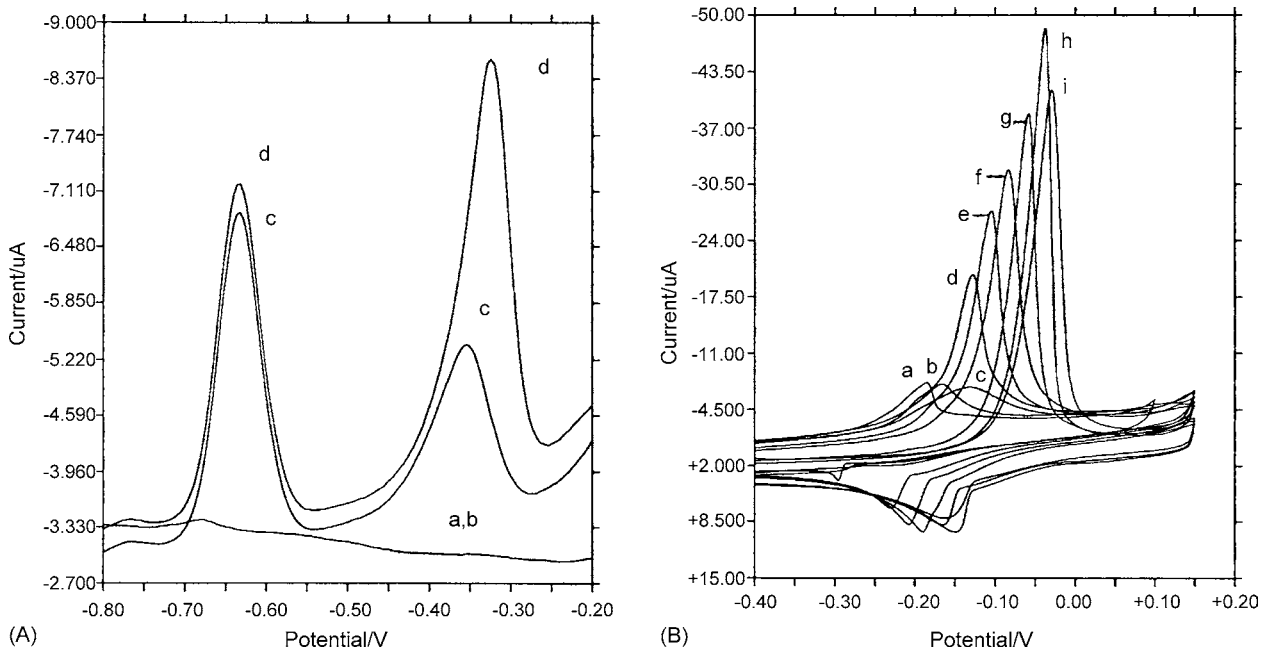


Fig. 6. (A) Comparison between the OSWS voltammograms of supporting electrolyte (peak a), (peak b) $a + 2.5 \times 10^{-8}$ M Cu(II), (peak c) $a + 5 \times 10^{-8}$ M (0.029 μg/ml) adriblastina, (peak d) $c + 2.5 \times 10^{-8}$ M Cu(II), at pH 7.4 onto in situ MFE after 30 s accumulation time. (B) Effect of pH on the cyclic voltammograms of 1×10^{-5} M adriblastina in the presence of 5×10^{-6} M Cu(II) at in situ MFE with scan rate 100 mV/s; pHs: (a) 2.4, (b) 3.36, (c) 4.36, (d) 5.35, (e) 6.45, (f) 7.4, (g) 7.92, (h) 8.51 and (i) 9.2.

−0.345 V). Therefore, OSWSV technique was used for all further experiments.

The electrochemical behavior of the Cu–adriblastina complex was also studied in the pH range 2.4–8.1 in HEPES buffer using cyclic voltammetry. Fig. 6B shows the effect of pH on the cyclic voltammograms of 1×10^{-5} M adriblastina in the presence of 5×10^{-6} M Cu(II) at in situ MFE. There is one oxidation/reduction pair; its current and potential values are highly dependent on pH values. Degree of irreversibility of the complex increased with increasing pHs and is highly dependent on scan rate values. The sites of complexation are generally considered to involve the C5-, C12-carbonyl groups and C6-, C11-phenolate oxygen in good agreement with the previously reported results by Greenaway and Dabrowiak using spectrophotometric method [40]. They pointed out that adriblastina forms two complexes—1:1 Cu(II)–adriblastina complex at low pH and 1:2 ratio at higher pHs. The relationship between scan rate and peak height gave linear relationship with slope tends to unity ($0.92\ \mu A\ s\ V^{-1}$) and correlation coefficient (0.9981) indicating an adsorption behaviour of the formed complex. The peaks also decreased rapidly upon repetitive scans with a little shift in potentials, indicating fast desorption of the complex molecules from electrode surface.

Effect of accumulation times on the OSWS voltammograms of Cu–adriblastina complex under the foregoing experimental parameters has been studied. It was found that linear relationships are obtained up to 300 s in the presence of 0.02 μg/ml adriblastina and decreased to be 60 s in the presence of 3.6 μg/ml. Stability of the complex was studied and it was found that the formed complex is stable for 4 days.

Double potential-step chronocoulometric studies of Cu–adriblastina complex at pH 7.4 have been investigated and the charge of the double layer (Q_{dl}) is calculated and found to be 3.5 μC for 1×10^{-7} M adriblastina giving surface coverage of 3.98×10^{-11} mol cm^{-2}. Thus, every one mole of adriblastina occupies an area of 1.23 nm^2 at in situ MFE surface.

Standard addition method is used for adriblastina quantitation in the presence of Cu(II) ions as complexing agents. Four accumulation times are used and compared with that without accumulation. The obtained data were summarized in Table 3. The stripping peak currents for the Cu–drug complex increase linearly with increasing drug concentrations up to 0.08 μM after low preconcentration times and other slopes observed after higher accumulation times; it may be attributed to the reorientation of complex molecules adsorbed on the electrode surface.

The reproducibility of the adsorption process is tested at adriblastina concentration of 5×10^{-8} M after 120 s. Well-defined reproducible peaks are obtained after running the voltammogram nine successive times with relative standard value of 1.9% showing a high reproducibility of the adsorption process. With accumulation time 240 s, adriblastina concentartion down to 1×10^{-11} M (5.8 pg/ml) could be easily assessed as a detection limit.

Table 3
Characteristic features of the calibration graphs for Cu–adriblastina complex onto in situ MFE, pH 7.4 in 0.05 M HEPES buffer

Concentration range (M)	$t_{acc.}$ (s)	Equation ($\mu A\ \mu M^{-1}$)	Limit of linearity (M)	Standard deviation	Correlation coefficient
1×10^{-10} to 8×10^{-8}	0	$Y = 0.39X$	8×10^{-8}	1.7177	0.8999
1×10^{-10} to 8×10^{-8}	30	$Y = 0.55X$	8×10^{-8}	1.7988	0.9786
1×10^{-10} to 8×10^{-8}	60	$Y = 0.62X + 0.09$	8×10^{-8}	1.8423	0.9571
1×10^{-10} to 8×10^{-8}	120	$Y = 0.77X + 3.1$	6×10^{-8}	1.9331	0.9476
1×10^{-10} to 8×10^{-8}	240	$Y = 0.81X + 3.5$	4×10^{-8}	1.9595	0.9578

3.6.2. Effect of Cu–adriblastina complex on ssDNA–adriblastina interaction

The complexation of anthracycline by metal ions is a route to get new compounds exhibiting lower toxicity comparing with that of the free drug [41]; the important point is that whether the stability of metal complexes is high enough in the presence of various biological molecules, whether they can reach the target without releasing the metal. Therefore, in the present work, the effect of copper ions, which essentially existed in the biological media, on the adriblastina–ssDNA interaction has been studied. Fig. 7 indicates a comparison between the OSWS voltammograms of HEPES buffer at pH 7.4 onto in situ MFE (peak a), 5×10^{-8} M adriblastina alone (peak b) and in the presence of 0.1 μg/ml ssDNA (peak c) after 60 s accumulation time. As previously discussed, the decreasing of peak (II) current value with little shifting in potential by adding ssDNA is due to the formation of hydrogen bonding between the oxidized form of the drug with ssDNA molecules. Peak (d) is obtained after adding

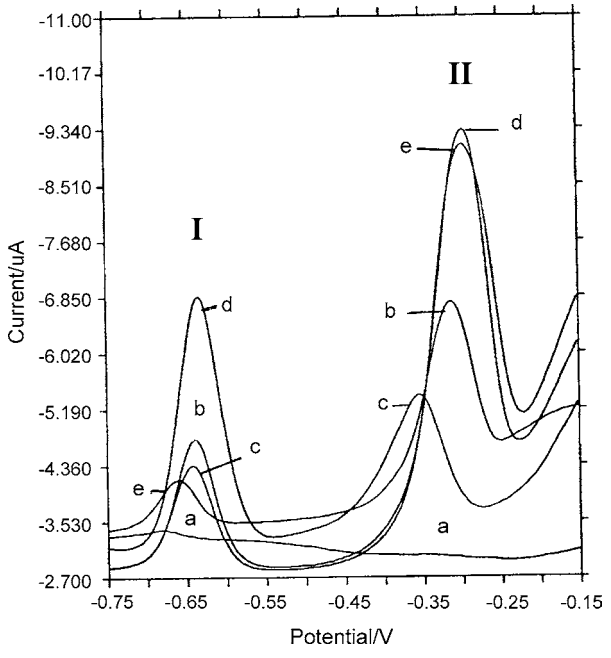


Fig. 7. Comparison between the OSWS voltammograms of HEPES buffer at pH 7.4 on in situ MFE as supporting electrolyte (peak a), (peak b) $a + 5 \times 10^{-8}$ M adriblastina, (peak c) $b + 0.1$ μg/ml ssDNA, (peak d) $b + 2.5 \times 10^{-8}$ M Cu(II) and (peak e) $d + 0.1$ μg/ml ssDNA after 60 s accumulation time.

2.5×10^{-8} M Cu(II) to adriblastina and the increase in peak current compared to drug alone is due to the formation of 1:2 Cu:adriblastina complex as discussed before. The effect of adding ssDNA on the Cu–drug complex is indicated by peak (e). There is a little decreasing in peak (II) current by adding ssDNA but this depression is throughout the reproducible range of peak running. Therefore, there is no interaction between the drug and ssDNA molecules in the presence of Cu(II) ions, i.e. the stability of Cu–drug complex is stronger than ssDNA–drug complex. So, the presence of metal ions in biological media may decrease the oxidative damage of ssDNA molecules when introducing adriblastina. The decrease in peak (I) current with little shifting in potential can be attributed to the presence of an adsorption competition at electrode surface. By adding different concentrations of ssDNA on 3×10^{-8} M (0.017 μg/ml) adriblastina in the presence of 1.5×10^{-8} M (0.952 ng/ml) Cu(II) at pH 7.4, there was an increase in peak (II) current of Cu–drug complex. This increment may be due to the formation of ion association complex between the positively charged Cu(II)–adriblastina binary complex and the negatively charged phosphate backbone of ssDNA, especially in higher concentrations of ssDNA (above 0.4 μg/ml). The electrochemical behaviour of Cu–adriblastina interacted with ssDNA have been also studied using cyclic voltammetry. An increment in oxidation peak current and in reduction peak current with shifting in potential is obtained. The redox peaks of the complex are highly dependent on the scan rate and the relation between scan rate and peak height gave a linear curve with slope 0.93 $\mu A\ s\ V^{-1}$ and correlation coefficient 0.9981 proving the adsorption character of the ion association complex.

Furthermore, classical least square and partial least square have been used for investigating the effect of adding Cu(II) ions and ssDNA molecules on the stability of their complexes with adriblastina. Least square equation of adriblastina–ssDNA complex was found to be $Y \pm 0.151 = (0.130 \pm 0.701)X - (0.121 \pm 0.020)$ with correlation coefficient ($r = 0.9994$). By introducing variant concentrations of copper ions, linear equation has been changed to be $Y \pm 0.051 = (0.601 \pm 0.205)X + (0.11 \pm 0.005)$ indicating the higher effect of adding copper ions on the formed complex. However, the addition of different concentrations of ssDNA on the Cu–adriblastina complex gave so small change in the slope value in that least square equation of Cu–adriblastina complex $Y \pm 0.151 = (0.620 \pm 0.070)X + (0.090 \pm 0.020)$

with correlation coefficient ($r = 0.9981$) has been changed to be $Y \pm 0.150 = (0.610 \pm 0.060)X + (0.091 \pm 0.010)$ with correlation coefficient ($r = 0.999$). These results confirm the higher stability of Cu–drug complex compared to ssDNA–drug complex.

3.7. Interferences and analytical utility for urine analysis

The effect of the possible interfering substances, which are of great significance in biological matrices, viz. ascorbic acid, uric acid, maltose, lactose and amino acids on the determination of adriblastina and Cu–adriblastina complex is tested onto in situ MFE and PtE. It was found that the addition of ascorbic acid and uric acid up to 1×10^{-4} and 1×10^{-3} M, respectively, has no effect. The sugar molecules such as maltose and lactose have no effect on drug determination. The addition of gelatin and some surfactants (e.g. CTAB, SDS and Triton X-100) which can interfere with drug determination by co-adsorption onto MFE indicates that there is a depression in peak current if their concentrations exceed 20 times drug concentration indicating the strong adsorption of drug and its complex at MFE or PtE. The influence of anions (e.g. SO_4^{2-}, NO_3^-, Cl^-) on the oxidation peaks of the investigated compound was studied. It was found that these anions have no influence on the determination of the drug up to 50 times the drug concentration.

The described method is suitable for the routine drug quantitation in biological fluids, viz. urine samples, due to the high adsorption character of the drug giving the best sensitivity and selectivity. As reported that adriblastina is available for intravenous use. The recommended dose is 60–75 mg/m^2, administered as a single rapid intravenous infusion; it is repeated after 21 days [1]. After oral administration, only about 25% of the drug reaches the circulation [7]. Less than 15% of the injected dose is excreted unchanged in the urine within 12–24 h. Fig. 8 shows the effect of accumulation time on representative voltammograms of 1 ml urine sample from patient injected with 100 mg adriblastina after dilution with supporting electrolyte to 10 ml. The unknown concentration of adriblastina has been calculated from the best fitting straight line for the drug addition at 60 s accumulation and compared with that calculated using single point standard method. The calculated concentration using linear fit equation is 0.723 ± 0.031 μM (the measurement error has been calculated by applying the principles of propagation of error) and the calculated one by single point standard method is 0.747 μM giving recovery value of $97 \pm 4\%$. These results indicate the high accuracy and validity of our analytical procedure in urine. Furthermore, the validity has been tested by applying *F*-test and *t*-test at the confidence limit 95% for the amount of adriblastina calculated by our analytical approach and that calculated by another reference HPLC method [42]. The calculated *F*-test and *t*-test were found to be 2.13 and 1.12, respectively, which are less than those tabulated with five number degrees of freedom (*n*) indicating that the measured error is due to random errors and not due to systematic errors. Adriblastina has been recovered in urine entire the range of 13–15% (w/w) of the injected drug. It was also found that down to 0.18 ng/ml could be easily detected in urine with 3.5% R.S.D.

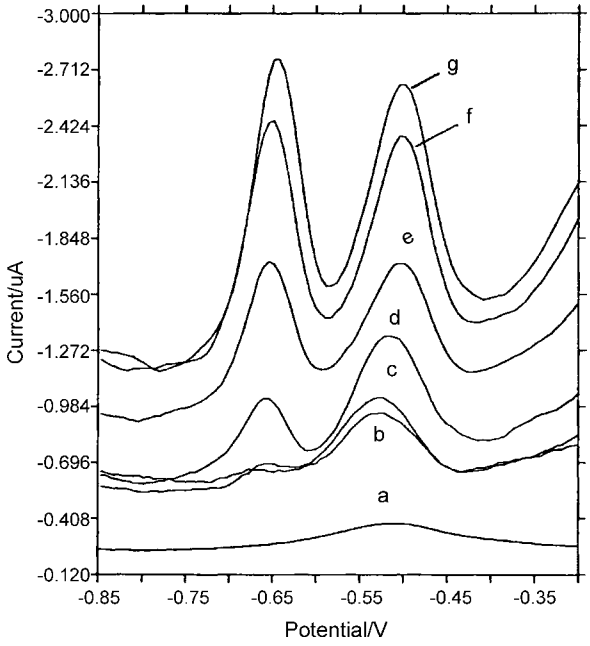


Fig. 8. Effect of t_{acc}. on the OSWS voltammogram of adriblastina in human urine (100 mg injected dose), other parameters are indicated in the text; (a) 0 s, (b) 30 s, (c) 60 s, (d) 120 s, (e) 180 s, (f) 240 s and (g) 300 s.

4. Conclusion

This work has demonstrated that adriblastina has three oxidation peaks and one reduction peak at in situ MFE and one oxidation peak and two reduction peaks at PtE. From the analytical point of view, the second oxidation peak could be easily used for the determination of nanomolar concentration levels of the drug in both aqueous and biological media at in situ MFE and the first reduction peak can be used for the quantitation of the drug at PtE. Also, our procedure explained the possible mechanism of the interaction between ssDNA and adriblastina at MFE which behaved like human membrane in the living cell as negatively charged surface [43]. The proposed method is sensitive, selective with excellent resolution, reproducible and because of its simplicity and reliability is particularly suitable for routine analysis. It allows for detecting down to 0.18 ng/ml adriblastina in urine samples. The complexation between Cu(II) and adriblastina was studied. It gives very sensitive and selective method for the determination of sub nanolevels of the drug in both aqueous and biological media. The stability of Cu–drug complex is higher than ssDNA–drug complex. Therefore, the presence of Cu(II) ions inhibits the oxidative damage of DNA molecules by introducing adriblastina.

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