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Voltammetric Studies of Cu-Adriblastina Complex and Its Effect on ssDNA-Adriblastina Interaction at *In Situ* Mercury Film Electrode

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Adriblastina, a cancerostatic anthracycline antibiotic, causes considerable oxidative damage to DNA molecules. The interaction of this compound with DNA was investigated using Osteryoung square wave stripping voltammetry (OSWSV) and cyclic voltammetry (CV) at an *in situ* mercury film electrode. It was found that the equilibrium constant of the bonded oxidized form of the drug was 63 times bigger more important than that of the bonded reduced form. Copper forms 1 metal: 2 drug stoichiometry complex which is highly stable compared to ssDNA-drug interaction and consequently inhibited the drug biochemical damaging effects. Copper complex offered sub-nanogram determination of adriblastina in aqueous and urine media.

Key words: Voltammetry, Copper complex, Adriblastina, ssDNA, Urine

INTRODUCTION

Adriblastina (doxorubicin hydrochloride) is one of the key 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-lyxohexopyranosyl) oxy-7,8,9,10-tetrahydro-6,8,11-trihydroxy-8-hydroxyacetyl-1-methoxy-5,12-naphthacenedione hydrochloride. The anthracycline antibiotics are currently experiencing wide clinical use in anticancer therapy (Hardman *et al.*, 1996). Adriblastina is effective against acute leukemias and malignant lymphomas. Also, it is active in a number of solid tumors, particularly breast cancer. It is an important ingredient for the successful treatment of ovarian, bladder, Hodgkinís and non Hodgkinís lymphomas, Wilmís tumor and neuroblastoma. Also, the drug is particularly beneficial in a wide range of sarcomas, including Osteogenic, Edwingís and soft tissue sarcomas. It has demonstrated activity in carcinomas of the endometrium, testes, prostate, cervix, head and neck (Hardman *et al.*, 1996). In addition, many new analogues and derivatives of adriblastina are being formulated and are coming to clinical trials.

A number of adriblastinaí's important biochemical properties have been described. Those properties could have a role in the therapeutic and toxic effects of the drug. The main biochemical properties are related to nucleic acid synthesis. Adriblastina can intercalate with DNA and this binding is considered responsible for the interference with template DNA function. The scission of the DNA is believed to be mediated either by the action of topoisomerase II or by the generation of free radicals (Hardman *et al.*, 1996). These reactions are highly destructive to the DNA and RNA. Adriblastina also can interact with cell membranes and alter their functions; this may play an important part in both the antitumor activities and the cardiac toxicity. Consequently, adriblastina is considered as an important model of anthracyclines for understanding the interaction of small molecules with the 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 find a mechanism to modify the aglycon moiety, and to generate new compounds, which may exhibit less toxicity.

The reported methods of analysis for the drug include high performance liquid chromatography (De Jong *et al.*, 1992; Alvarez-Cedron *et al.*, 1999; Buehler *et al.*, 1999) and UV-Vis spectrophotometry (Sastry and Rao, 1996). Adriblastina was determined by a.c. polarography (Golabi and Nematollahi, 1992), differential-pulse polarography (Sternson and Thomas, 1977) and adsorptive stripping voltammetry at mercury electrodes (Hahn and Lee, 2004).

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There are few procedures for the voltammetric determination of the drug at the solid electrodes (Hu *et al.*, 1999; Chaney and Baldwin, 1982). Literatures on the voltammetric studies of metal-adriblastina complexes and their effects on DNA-drug interaction are almost inexistent.

Therefore, the aim of the present work was to study the possible complexation reaction between Cu(II) ions and adriblastina, and its effect on the drug-ssDNA interaction at *in situ*-mercury film electrode (in-situ MFE). The developed method was applied for the determination of adriblastina in urine samples.

MATERIALS AND METHODS

Reagents and solutions

A fresh solution of adriblastina was prepared daily in doubly distilled water. The solutions were diluted as required for standard additions. The adriblastina used was provided by Pharmacia & Upjohn S.P.A company, Milan-Italy. It was used without any further purification. Different types of supporting electrolytes such as phosphoric acid, acetate, borate, Britton-Robinson, isotonic Sorensen and HEPES buffers were used. HEPES buffer (0.05 M) was the selected supporting electrolyte that gave the best signals. A stock solution of mercuric ion (10^{-2} mol/L) was prepared by dissolving the required weight of basic mercuric nitrate (May & Baker LTD., Dagenham, England) in doubly distilled water. The 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 modified version of the method of Alberts and Herrick using calf thymus DNA, D 1501. The resulting solution was divided into ten aliquots (for daily use) and was kept frozen in polyethylene vials. One mg of ssDNA is equivalent to approximately 25 A_{260} units. Solutions of diverse ions were prepared using the nitrate, sulphate or perchlorate salts of the metal ions (AnalaR products). Dilute acids were added to prevent hydrolysis whenever needed. Urine samples were taken from patients undergoing adriblastina chemotherapy. The preparation of urine samples for the drug determination was made as reported in a previous work (El-Maali, 2000). All other substances were graded.

Instruments

Voltammetric measurements were recorded using the CV-50W Voltammetric Analyzer (USA) electrochemical running under windows™ software. All controlled parameters were entered through a BAS/windows interface. This information was transferred to the CV-50W microprocessor, where optimum hardware settings were calculated for the specified technique. These values were loaded automatically and upon applying the command run, the data were collected and transmitted to the PC where they were displayed in virtual real time. The standard C-2 cell stand was fully shielded in a Faraday cage (EF-1080) with three electrodes: a glassy carbon (MF-2012, diameter 3 mm) working electrode, a silver/silver chloride reference electrode (MF-2063) and a platinum wire auxiliary electrode (MW-1032). Voltammograms were collected using Hewlett Packard laser jet 4 L printer. These data were sometimes plotted using Microsoft Excel program utilizing either the line or scatter presentation. The pH were adjusted using a Fisher Scientific Accument pH meter Model 810, equipped with a combined glass electrode. The pH meter was 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.

Procedure

A 10 mL volume containing HEPES buffer (0.05 M) as supporting electrolyte was added to the cell and degassed for 8 min with highly purified nitrogen. 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 the mercury and the measured complex. The deposition was achieved by applying high voltage (-900 mV). The deposited material was then stripped from the mercury thin film by scanning the Osteryoung square-wave potential. This electrode combines the sensitivity of thin films with high selectivity and reproducibility (Abd El-Hady *et al.*, 2004). After the analysis, the electrode surface was easily polished by wiping it with a BAS cloth disk coated with two drops of alumina (CF-1050) and doubly distilled water. Voltammograms were recorded after a definite quiet time by applying Osteryoung square-wave potential scan from -900 to -100 mV at pH 7.4. The solution was stirred up using a stirring magnet (ER-9132) at a constant stirring rate (100 rpm). The interaction of ssDNA with adriblastina was studied by titration of different ssDNA concentrations with a fixed concentration of the drug and vice versa. The effect of the Cu-adriblastina complex on the ssDNA-drug interaction was studied. All the tests were operated at room temperature (25±2°C).

RESULTS AND DISCUSSION

A growing body of evidence suggests that the pharmacological activity (Sugioka and Nakano, 1982) of adriblastina is related to metal ions. As metal ions presence in all biological processes involving nucleic acids, the occurrence of metal chelates with adriblastina inside the cell may be an important factor of its toxicity inhibition. On the other hand, the cardiotoxicity of the drug has prompted

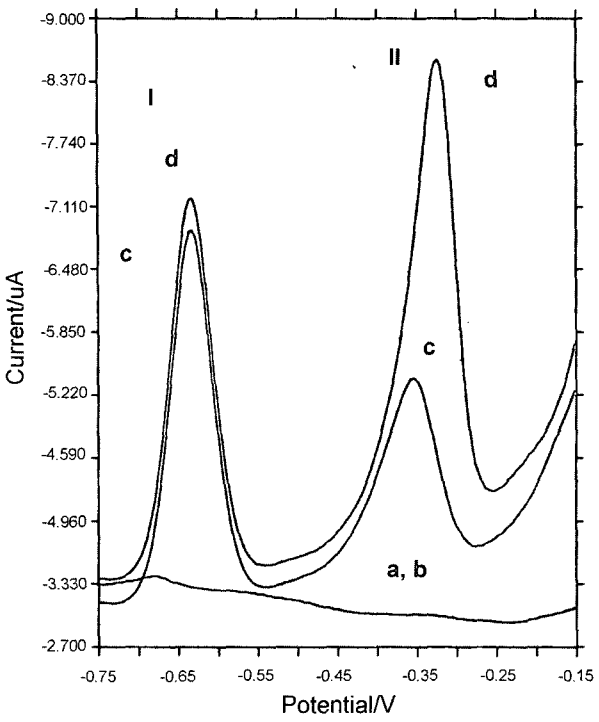

Fig. 1. Comparison between the OSWS voltammograms of supporting electrolyte (peak a), b) a + 2.5×10^{-8} M Cu(II), c) a +5×10^{-8} M (0.029 μg/mL) adriblastina, d) c + 2.5×10^{-8} M Cu(II), at pH 7.4 at in-situ MFE after 30 s accumulation time.

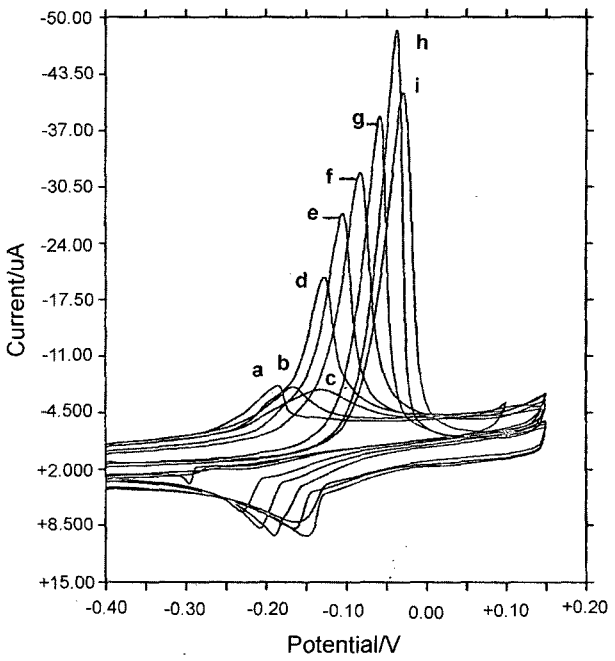

Fig. 2. The effect of pH on cyclic voltammograms of 1×10^{-5} M adriblastina in the presence of 5×10^{-6} M Cu(II) at in-situ MFE surface with scan rate 100 mV/s; pH: a) 2.4, b) 3.36, c) 4.36, d) 5.35, e) 6.45, f) 7.4, g) 7.92, h) 8.51, and i) 9.2.

us to search for a noncardiotoxic yet active species. The complexation of adriblastina by metal ions appears to be a route to modify the aglycon moiety, and to get new compounds which may exhibit less toxicity. Therefore, it is very important to investigate the electrochemical characteristic of the metal-adriblastina complexes. Among several possible essential and non-essential metal ions viz. Cu(II), Fe(III), Fe(II), Zn(II), Ca(II), and 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 (OSWSV) were used for such study.

Preliminary tests were done for adriblastina-Cu(II) complex with an interfacial accumulation behavior at in-situ MFE. The nature and concentration of supporting electrolyte and the pH of the solution strongly affected the electrochemical behavior of the complex, its sensitivity and its selectivity. The best signals were obtained in the presence of 0.05 M HEPES buffer. Fig. 1 shows a comparison between Osteryoung square wave stripping (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 (peak c) and presence of Cu(II) ions (peak d). The increment in the signal of Cu-adriblastina peak was about 2.5 times that of adriblastina alone, with a little shift to more positive value in potential (about 31 mV). The shift in value indicated the formation of the Cu-adriblastina complex.

The electrochemical behavior of the Cu-adriblastina complex was studied in the pH range of 2.4-8.1 using cyclic voltammetry. Fig. 2 shows the effect of pH on the cyclic voltammograms of 1×0^{-5} M adriblastina in the presence of 5×10^{-6} M Cu(II). There was one oxidation/reduction pair, which current and potential values were highly dependent on the pH value. The degree of irreversibility of the complex increased with increasing pH values, and it was highly dependent on the scan rate value. The scan rate and the peak height had a linear relationhip with a slope value that tended to unity (0.92 μA s V^{-1}) and a correlation coefficient of 0.9981. This indicated the adsorption behaviour of the formed complex. The peaks also decreased rapidly upon repetitive scans with a little shift in potentials, which indicated a fast desorption of the complex molecules from the electrode surface.

The effect of operating parameters, such as wave form, pulse amplitude, frequency, step E, and stirring rate on the peak response was investigated between 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 a 120 s accumulation. The OSWS voltammogram had a peak current value of 4.6 μA at about -0.32 V, which was more sensitive than LSS voltammogram (0.8 μA at -0.31 V) and DPS voltammogram (0.4 μA at -0.345 V). Therefore, the OSWSV technique was used

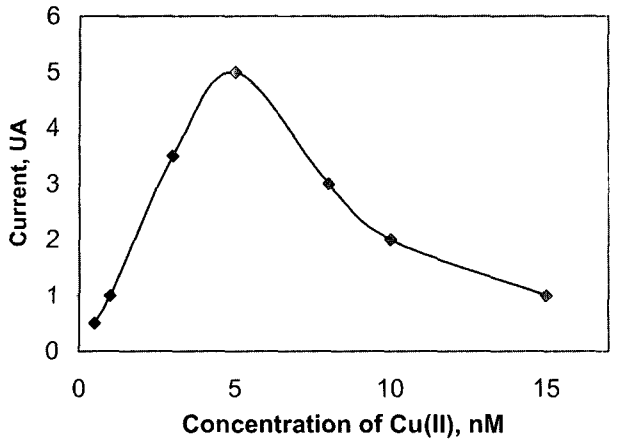

Fig. 3. The effect of Cu(II) ions ranged from 1×10^{-9} to 1.5×10^{-8} M on the OSWS voltammograms of 1×10^{-8} M adriblastina onto in-situ MFE surface after 60 s accumulation in the presence of 0.05 M HEPES as supporting electrolyte, pH 7.4, potential range -900 to -100 mV, amplitude 15 mV, frequency 160 Hz, quiet time 0 s, step potential 6 mV and stirring rate 200 rpm.

for all remaining experiments, under the following optimal conditions: square wave amplitude 15 mV, square wave frequency 160 Hz, quiet time 0 s, step potential 6 mV and stirring rate 200 rpm.

The effect of Cu(II) concentrations on the sensitivity of its complex with adriblastina was investigated. The sites of complexation were generally considered to involve carbonyl groups and phenolate oxygen, as previously reported by Greenaway *et al.* (1982) using the spectrophotometric method. They pointed out that adriblastina forms two complexes: 1:1 Cu(II)-adriblastina at low pH, and 1:2 ratio at higher pHs. Fig. 3 shows the effect of Cu(II) ions addition on the OSWS voltammograms of adriblastina. It was observed that the best sensitivity achieved in the presence of 1:2 (Cu:adriblastina concentration ratio) elucidating the stoichiometry of the complex formed.

The effect of the accumulation time (Fig. 4A) on the OSWS voltammograms of 2.9 ng/mL (5×10^{-9} M) adriblastina in the presence of 2.5×10^{-9} M Cu(II) ions, HEPES buffer at pH 7.4 under the foregoing experimental parameters was achieved. The linearity limits of time at different concentrations of the complex were studied. The linear relationships extended up to 300 s in the presence of 0.02 μg/mL adriblastina, but was limited to 60 s in the presence of 3.6 μg/mL. A test on the stability of the complex revealed that it was stable during 4 days.

Double potential-step chronocoulometric studies of the Cu-adriblastina complex at pH 7.4 was investigated. The charge of the double layer (Q_{dl}) was calculated and found to be 3.5 μC for 1×10^{-7} M adriblastina. The surface coverage can be measured from the division of the number of coulombs transferred by the conversion quantity (nFA)

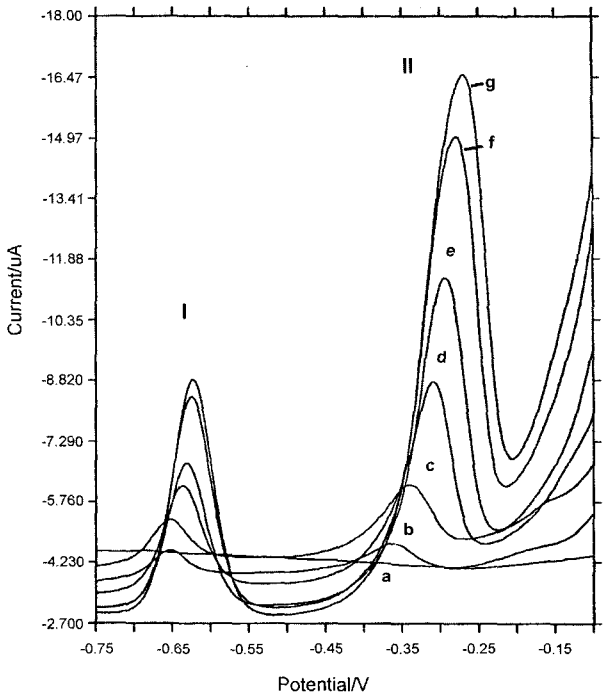

Fig. 4A. The effect of accumulation time on the OSWS voltammograms of 2.9 ng/mL (5×10^{-9} M) adriblastina in the presence of 2.5×10^{-9} M Cu(II) ions under the foregoing experimental parameters as cited in Fig. 3; a) 0, b) 30, c) 60, d) 120, e) 180, f) 240, and g) 300 s.

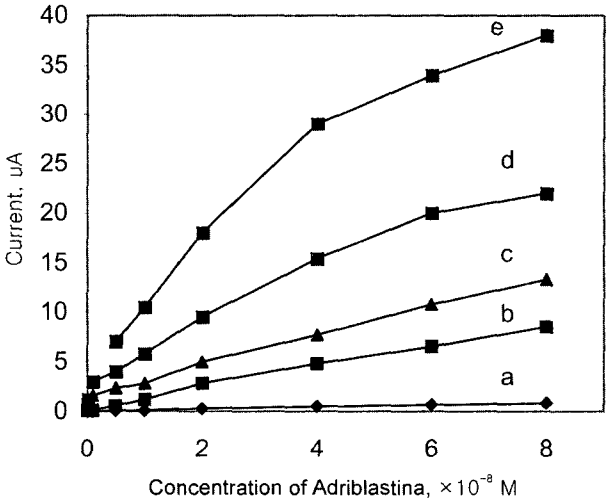

Fig. 4B. Calibration plots of different adriblastina and Cu(II) ions spikes from 0.1 nM to 0.08 μM with anodic potential scan at pH 7.4 following 0 (a), 30 (b), 60 (c), 120 (d), and 240 (e) seconds of preconcentration. Other parameters as cited in Fig. 3.

yielding coverage of 3.98×10^{-11} mol cm^{-2}. Thus, every mole occupied an area of 1.23 nm^2 at *in situ* MFE surface.

The standard addition method was used for adriblastina quantitation in aqueous medium in the presence of Cu(II) ions as complexing agents. Four accumulation times were used and compared with those without accumulation. These calibrations are shown in Fig. 4B, and their calcul-

Table I. Characteristic features of the calibration graphs for Cu-adriblastina complex at in-situ MFE, pH 7.4 in HEPES buffer

Concentration range (M)	$t_{acc.}$ (s)	Equation (μA $μM^{-1}$)	Limit of linearity (M)	Standard deviation	Correlation coefficient
1×10^{-10}-8×10^{-8}	0	Y = 0.39X	8×10^{-8}	1.7177	0.9899
1×10^{-10}-8×10^{-8}	30	Y = 0.55X	8×10^{-8}	1.7988	0.9786
1×10^{-10}-8×10^{-8}	60	Y = 0.62X+0.9	8×10^{-8}	1.8423	0.9571
1×10^{-10}-8×10^{-8}	120	Y = 0.77X + 3.1	6×10^{-8}	1.9331	0.9476
1×10^{-10}-8×10^{-8}	240	Y = 0.81X + 3.5	4×10^{-8}	1.9595	0.9578

ations are summarized in Table I. The stripping peak currents for the Cu-drug complex increased linearly with increasing drug concentrations up to 0.08 μM after low preconcentration times and other slopes observed after higher accumulation times; this may be attributed to the reorientation of the complex molecules adsorbed on the electrode surface. The reproducibility of the adsorption process was tested at adriblastina's concentration limit of 5×10^{-8} M after 120 s. Well-defined reproducible peaks were obtained after running the voltammogram nine successive times. A relative standard value (RSD) of 1.9% was reported 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 (signal-to-noise ratio 3) with 3.2% RSD.

Electrochemical studies on the interaction of adriblastina to ssDNA

The electrochemical investigation of the possible interaction of adriblastina with ssDNA under the foregoing experimental conditions was studied. It was found that the first oxidation peak height for adriblastina decreased after the addition of ssDNA molecules; this decrease may be due to the adsorptive competition between ssDNA molecules and the drug molecules. There was also one-third depression in the second oxidation peak after having added 1.0 μg/mL ssDNA with shifting in potential (about 48 mV to more negative potential). This depression may be due to the decreasing in the diffusion coefficient of adriblastina when it interacted with ssDNA molecules. It is well-known that the interactions between anthracycline moieties and DNA bases are due to hydrogen bonding between certain groups in the drug molecules and on the DNA bases or electrostatic attractions (Hardman *et al.*, 1996). Therefore, we postulated that the interaction mechanism between ssDNA and adriblastina may be due to the formation of hydrogen bond between the -C=O group in the oxidized form of the drug and the $-NH_2$ group in the guanine moiety. Another explanation is that the DNA-drug interaction could be attributed to the electrostatic interaction between the protonated amine group of the 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 neutral one.

The formal potentials of adriblastina in the absence of ssDNA (E^o) and presence of ssDNA ($E^{o\prime}$) was calculated by applying cyclic voltammetry with scan rate 200 mV/s. A positive shift in the formal potential (about 53 mV) was determined. Then the ratio of the equilibrium constant (K_1) and the equilibrium constant (K_2) form by applying the equation below (Abd El-Hady *et al.*, 2004) was claculated:

$$2(E^{o\prime} - E^o) = 0.059 \log (K_2/K_1)$$

K_2 was 63 times bigger than K_1. This means that ssDNA highly interacted with the oxidized form, which contains a carbonyl group and weakly interacted with the reduced form, which contains a hydroxyl group. Therefore, it was concluded that the carbonyl group in the oxidized form was the predominant functional group for the interaction between ssDNA and adriblastina. The effect of the scan rate on the cyclic voltammograms of the drug-ssDNA interaction was studied. The relationship between the scan rate and the peak heights gave a linear curve with a slope value of 0.91 μA s V^{-1} and a correlation coefficient of 0.9983 indicating an adsorption behaviour of the adriblastina-ssDNA complex.

Effect of the Cu-adriblastina complex on the ssDNA-adriblastina interaction

The complexation of anthracycline by metal ions is a route to get new compounds exhibiting lower toxicity compared to that of free drugs. The important issue is the stability of the metal complexes in the presence of various biological molecules, and their capability to reach the target without releasing the metal. Therefore, in the present work, the role of copper ions, which essentially exist in the biological media, on the adriblastina-ssDNA interaction was studied. Fig. 5 illustrates a comparison of the OSWS voltammograms of HEPES buffer at pH 7.4 at in-situ MFE (peak a), 5×10^{-8} M adriblastina in the absence of ssDNA (peak b) and 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 after adding ssDNA may be due to the formation

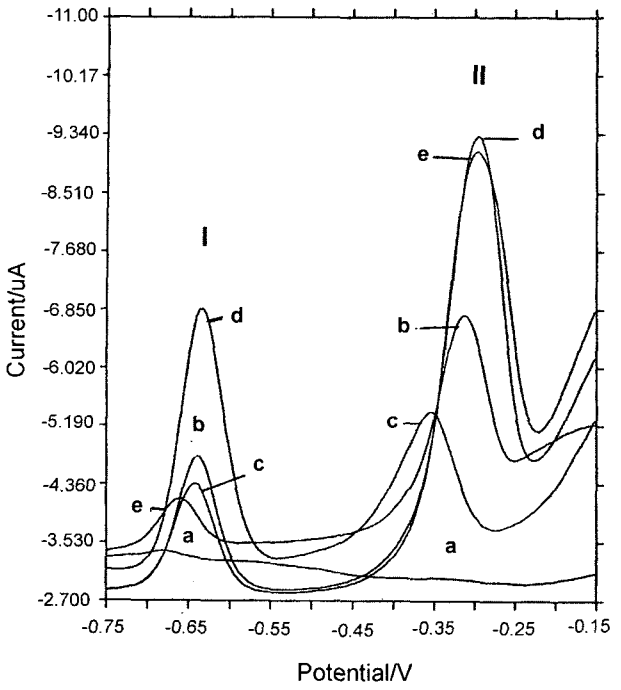

Fig. 5. Comparison between the OSWS voltammograms of HEPES buffer at pH 7.4 at in-situ MFE as supporting electrolyte (peak a), b) a + 5×10^{-8} M adriblastina, c) b + 0.1 μg/mL ssDNA, d) b + 2.5×10^{-8} M Cu(II), and e) d + 0.1 μg/mL ssDNA after 60 s accumulation time.

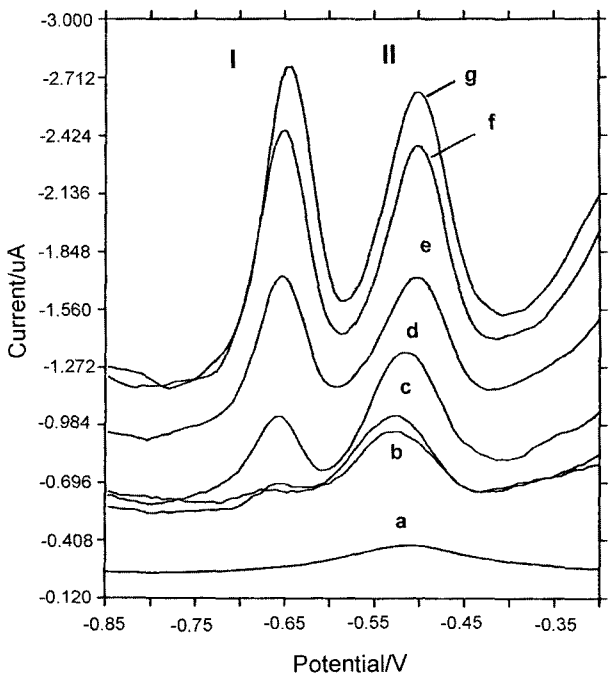

Fig. 6. *The effect of* t_{acc}. *on OSWS voltammogram of adriblastina* analysis in human urine after injection of 100 mg, other parameters are indicated in the text; a) 0, b) 30, c) 60, d) 120, e) 180, f) 240, and g) 300 s.

of hydrogen bonds between the oxidized form of the drug and ssDNA molecules. Peak d was obtained after the addition of 2.5×10^{-8} M Cu(II) ions to adriblastina and the increasing in peak current may be due to the formation of 1Cu:2adriblastina complex as discussed before. The effect of adding ssDNA on the Cu-drug complex is indicated by peak e. The decrease in peak (II) current after the addition of ssDNA was limited but this depression was through the reproducible range of peak running. Therefore, there was no interaction between the drug and ssDNA molecules in the presence of Cu(II) ions, i.e. the stability of the Cu-drug complex was stronger than that of the ssDNA-drug complex. Therefore, the presence of metal ions in biological media may decrease the oxidative damage of ssDNA molecules when introducing adriblastina. The decreasing in peak (I) current with little shifting in potential can be attributed to the competition at the MFE surface between the adsorbed ssDNA molecules and the adsorbed Cu-adriblastina complex. The addition of high concentrations of ssDNA (more than 0.4 μg/mL) 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, increased the peak (II) current of the Cu-drug complex. This increment may be due to the formation of ion complexes between the positively charged Cu(II)-adriblastina binary complex and the negatively charged phosphate groups of the ssDNA molecules. The electrochemical interaction of Cu-adriblastina with ssDNA was also studied using cyclic voltammetry. An increment in oxidation/reduction peak currents with shifting in potential was obtained. The redox peaks of the complex were highly dependent on the scan rate and the relation between the scan rate and the peak height gave a linear curve with a slope value of 0.93 μAsV^{-1} and a correlation coefficient of 0.9981 proving the adsorption character of the ion association complex.

Interferences and analytical utility for urine analysis

The effect of the probable interfering substances, which is of great significance in biological matrices viz. ascorbic acid, uric acid, maltose, lactose and amino acids, on Cu-adriblastina complex was tested. It was found that the addition of ascorbic acid and uric acid up to 1×0^{-4} and 1×10^{-3} M, respectively had no effect. Also, sugar molecules such as maltose and lactose had no effect on the drug determination. The addition of gelatin and some surfactants (e.g. CTAB, SDS and Triton X-100) that can interfere with the drug determination, by co-adsorption onto MFE, indicated that there was a slight depression in peak current when their concentrations exceeded about 20 times the drug's concentration. The influence of anions (e.g. SO_4^{2-}, NO_3^-, Cl^-) and metal cations was studied. It was found that up to 50 times drug concentration, there was no influence.

The described method is suitable for the routine drug

quantitation in biological fluids like urine samples because the high adsorption character of the formed complex gave the best sensitivity and selectivity. As reported, adriblastina is available for intravenous use. The recommended dose is 60 to 75 mg/m^2. It is administered as a single rapid intravenous infusion, and is repeated after 21 days (Hardman *et al.*, 1996). Fig. 6 shows the effect of accumulation time on 1 mL urine sample from patient injected with 100 mg adriblastina after dilution with supporting electrolyte to 10 mL. It was found that 13-15% of the injected drug dose was recovered in urine samples in good agreement with previously documented results (Chaney and Baldwin, 1985; De Jong *et al.*, 1992). The concentration of adriblastina was determined by the single point standard method, which is not different from the calibration method (F-test and *t*-test at 95% confidence level). This indicates that urine medium did not affect the determination of adriblastina but led to some shifting in potentials. This may be due to the co-adsorption of some organic species that exist in urine. In addition, the amount of 0.18 ng/mL could be easily detected in urine samples.

It was concluded that adriblastina reacted with Cu(II) ions giving a 1:2 metal:drug complex in HEPES buffer at pH 7.4, and the measurement of complex formation seemed to be a very sensitive and selective method for the determination of sub nanolevels of the drug in both aqueous and biological media. It allowed for the detection of 0.18 ng/mL adriblastina in urine samples. The Cu-drug complex was more stable than the ssDNA-drug complex. Therefore, it was deduced that the presence of Cu(II) ions inhibited the oxidative damage of DNA molecules when adriblastina was introduced.

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