ELECTROCHEMICAL SENSOR FOR THE SIMULTANEOUS VOLTAMMETRIC DETERMINATION OF ADENOSINE AND ADENINE

A DISSERTATION

Submitted in partial fulfillment of the requirements for the award of the degree of

MASTER OF TECHNOLOGY

in

ADVANCED CHEMICAL ANALYSIS

By

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **"ELECTROCHEMICAL SENSOR FOR THE DETERMINATION OF ADENOSINE AND ADENINE"** in partial fulfillment of the requirement for the award of the Degree of **Master of Technology** in **Advanced Chemical Analysis** submitted in the Department of Chemistry, Indian Institute of Technology Roorkee, Roorkee, is an authentic record of my own work carried out during a period of July, 2010 to June, 2011 under the supervision of Dr. R.N. Goyal, Professor, Department of Chemistry, Indian Institute of Technology Roorkee, Roorkee.

The matter embodied in this thesis has not been submitted by me for the award of any other degree.

Rakesh Kumar Singh

Date: 24th June, 2011 **(RAKESH KUMAR SINGH)**

This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

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ABSTRACT

Electrochemical method based on voltammetry using bare and single walled carbon nanotubes (SWNT) modified edge plane pyrolytic graphite electrode (EPPGE) is proposed for the simultaneous determination of adenosine and adenine in phosphate buffer medium of pH 7.2. The oxidation peak current of both the analytes increased significantly along with the negative shift of peak potentials using SWNT/EPPGE as compared to bare EPPGE. The modified electrode exhibited enhanced electrocatalytic properties towards oxidation of adenosine and adenine with a peak potential of ~ 1190 mV and ~ 856 mV, respectively. The oxidation of both the compounds occurred in a pH dependent process, and the electrode reaction followed diffusion controlled pathway. The concentrations versus peak current plots were linear for both the analytes and the detection limit (3σ / slope) observed for adenine and adenosine were 1.1 and 2.9 μM, respectively. The developed protocol is implemented for the simultaneous determination of adenosine and adenine in human urine samples of normal subjects.

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Rakesh Kumar Singh

Place: Roorkee

RAKESH KUMAR SINGH

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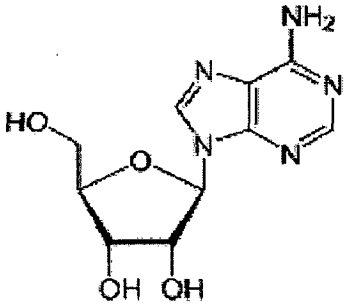
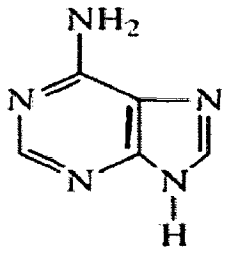
Chapter-1
INTRODUCTION

Adenosine (I) being a purine nucleosides performs many important functions in human body and biological processes [1]. It modulates physiological functions in heart and brain, regulates oxygen supply during cell stress and plays important role in the regulation of renal function [2, 3]. It is a potent anti-inflammatory and anti-arrhythmic agent which is important in the control of coronary and cerebral blood flow [4-6]. It is an inhibitory neurotransmitter [7, 8] which imparts a role in promoting sleep and suppressing arousal [9]. It has anti-diuretic effect, anti-epileptic effects on the central nervous system and anti-secretary effect in the stomach [10]. Adenosine is an endogenous vasodilating agent [11] and also administered for the treatment of gastrointestinal diseases in many cases [12]. The amounts of adenosine in the urine and plasma samples are considered be the marker of some diseases such as carcinoma or liver diseases [13]. Hence, it is considered important to determine the concentration of adenosine in different human body fluids samples.

Adenine (II) is one of the two purine nucleobases and is therefore essential molecules of life and evolution. Adenine is of tremendous biological significance since, it is one of the nitrogenous bases found in deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) to make up genetic information. It is a component of adenosine triphosphate (ATP) which is a major energy releasing molecule in cells. Adenine is also a part of various coenzymes and being a part of nucleic acids it imparts a central role in protein synthesis.



(I)



(II)

1.1 LITERATURE SURVEY

Different analytical methods have been proposed for the determination of adenosine and most of them are based on the chromatographic techniques, which is sensitive and accurate with requirement of some pretreatment procedures [14, 15]. In view of the importance of purine nucleosides in human physiology, various attempts have been made to determine them individually in body fluids by different techniques [16-17] and a concentration as low as 10^{-8} M/L have been determined. The electrooxidation of adenosine using the common working electrode showed high oxidation potential and limited sensitivity. At many solid electrodes even the peak did not appear as it merged with the background. Hence, the chemically modified electrodes were used to overcome the disadvantages and improved the sensitivity and detection limit for adenosine [18-19].

Ping *et al.* [20] determined seven nucleobases and nucleosides in tuber samples by dispersive solid phase extraction (DSPE) combined with liquid chromatography-mass spectrometry. The D 3520 macroporus resin was used as the DSPE sorbent and good sensitivity was observed. The reverse phase HPLC method was used by Wang *et al.* for the determination of various purines and their ribosides in *Fritillaria anhuiensis*. The detection wavelength used was 260 nm at a flow rate of 1.0 ml/mm using methanol-water as mobile phase [21]. The simultaneous HPLC determination of adenine, uridine,

guanosine and adenosine in six traditional Chinese medicines was reported by Zhang *et al.* [22]. The diode array detection was carried out at 260 nm and nM detection limits were observed. Later the same purine derivatives were analyzed by reverse phase HPLC [23] at XB-C_{18} column using ammonium acetate-acetic acid buffer (pH 4.30) and methanol as mobile phases. The better detection limit over earlier studies [22] was noticed in this case. A sensitive, selective and reliable Liquid Chromatography-Mass Spectrometry coupled with electrospray ionization interface method has been reported for the simultaneous separation and determination of thymine, adenine, adenosine and *cordycepin* in *cordyceps sinensis*. The internal standard used was 2-chloroadenosine and $[M+H]^+$ ions at 127, 136, 268, 252 and 302 were chosen for quantitative analysis of nucleosides [24]. Zhou *et al.* [25] also determined adenine and adenosine in *cordyceps sinensis* at an electrospray ionization interface. The linear regression equations for adenine and adenosine were found as:

$$Y = 0.07264\ X + 0.00622 \qquad \text{for adenine}$$

$$Y = 0.15970\ X + 0.0146 \qquad \text{for adenosine}$$

with correlation coefficients 0.9987 and 0.9991, respectively. The determination of twelve nucleosides and nucleobases in different species of *cordyceps* has also been carried out by Yang *et al.* [26] using capillary electrophoresis-mass spectrometry (CE-MS). The optimum CE electrolyte was found as 100 mM formic acid.

Purine nucleosides have also been determined in plasma samples from patients with diabetic nephropathy using HPLC-UV/MS/MS [27]. Both UV and MS detectors were used for quantification. The limit of detection for adenine was found in ng/mL range. The intraday and interday relative standard deviations (RSD) were lower than 15%. The simultaneous determination of adenosine triphosphate and its metabolites in human whole blood by RP-HPLC and UV-detection has been reported by Coolen *et al.* [28]. The lower detection limit for all adenine derivatives was below 0.3 μmol/L and the method

demonstrated good linearity. The simultaneous determination of adenine and adenosine with five more nucleobases has been reported by Yuan *et al.* [29] in edible fungi using a gradient reverse phase HPLC. The analysis showed that total nucleosides and nucleobases content ranged from 0.14 to 26.57 mg/g dry matter in these fungi.

HPLC has been used by Gu *et al.* [30] for the simultaneous determination of purine nucleosides and bases in *cordyceps sp. fermn.* preparations. The method was found suitable for successful determination to analyze the compounds. A rapid ultra-performance liquid chromatography (UPLC) was developed for simultaneous determination of 14 nucleosides and nucleobases including adenine and adenosine. The separation was performed at UPLC BEH C_{18} column and separation was achieved in 5 min. [31]. A capillary zone electrophoresis (CZE) and a HPLC method was used by Ying *et al.* [32] for the determination of adenine, adenosine and uracil in natural *cordyceps sinensis* and cultured *cordyceps mycelia*. An uncoated fused-silica capillary was used and nM concentrations were determined. In another method LC/ESI-MS was used for the simultaneous determination of adenine, adenosine and uridine in *cordyceps sinensis*. Selective ion monitoring mode was used and monitoring was done at m/e 136 for adenine and 268 for adenosine [33]. Katayama *et al.* [34] determined six adenyl purines in human plasma by using HPLC with fluorescence derivatization. Each derivatized purine was separated on a Capcell pack SG120A column and detection limits were found in the range 100-1000 fm.

The studies on compounds that catalyze depurination of adenosine to release adenine from nucleic acids has also been reported by Schramm [35]. The fluorescence enhancement of adenine by addition of heavy atom containing molecule such as 3-bromopropane-1-ol, 6-methyl purine etc. has been reported in literature [36]. Adenosine

did not exhibit any enhancement and the effect of temperature on fluorescence enhancement for adenine has also been studied. A review on role of Adenine phosphoribosyltransferase (APRT) in enzymopathy of purine metabolism was presented by Bouzidi *et al.* [37]. APRT is a salvage enzyme that normally catalyzes the conversion of adenine to adenosine monophosphate. The APRT deficiency results in adenine accumulation in the form of 2, 8-dihydroxyadenine to form stones and ultimately may cause renal failure. Peterson *et al.* [38] has found that in *E. Coli*, the conversion of adenine to guanine nucleotides occurs via adenosine intermediate. The cryptic adenine deaminase gene of *Escherichia coli* was found responsible for the conversion. Adenosine produced by the enzymic conversion of adenine nucleotides has been found to produce analgesia in the dorsal horn of the rat spinal cord [39]. The conversion of adenine to adenosine has been reported by various intermedius of staphylococcus [40]. This conversion has been used for differentiation of *S. Intermedius* from other *coagulase-pos. staphylococci.* The immune deficiency associated with adenosine deaminase deficiency has been related to increased amounts of adenine and adenosine in erythrocytes, plasma and urine of a 5-month old infant [41]. The adenine and adenosine were measured using ion exchange separation technique and were found as 2.0 μM, 3.0 μM in plasma, 86 and 1 μM in erythrocytes and 82 μM in urine. The corresponding values in controls were 0.6, 0.3 in plasma, 13 and 1 μM in erythrocytes and adenine was not detectable in urine.

Thus, it is clear from the above paragraphs that determination of adenine and adenosine has attracted considerable attention. However, most of the work is reported by using HPLC, LC-ESI/MS and related techniques, which are expensive and require derivatization. During the last few years several electrochemical methods have been developed for the individual determination of inosine, guanosine, guanine, adenosine and

adenine [42-46]. However, simultaneous determination of adenosine and adenine has attracted little attention using voltammetric techniques. The chromatographic methods have high operational cost and involve time consuming extraction steps. The oxidation of adenosine occurs at high positive potential and there can be a chance of overlapping of adenosine potential with the potentials at which supporting electrolytes get discharged using conventional techniques and electrodes [47]. To the best of our knowledge, only one paper is reported on the simultaneous determination of adenosine and adenine using polarography [48]. The addition of adenosine was found to decrease the peak of adenine as both had similar $E_{1/2}$ values. The electro oxidation of these compounds require a relatively high oxidation potential at conventional electrodes which results in the merging of the signal current with the background discharge current. Carbon nanotubes (CNTs) with their extraordinary mechanical and unique electrochemical properties have garnered much attention in the past five years. The CNTs based electrodes are generally prepared by casting the suspension of CNTs on conventional electrode surfaces [49-52]. In the present study, single wall carbon nanotube (SWNT) modified edge plane pyrolytic graphite electrode (EPPGE) has been used for the simultaneous determination of adenosine and adenine using square wave voltammetric (SWV) procedure.

Chapter-2

EXPERIMENTAL

2.1 REAGENTS

Adenosine was obtained from Merck (Germany) and adenine was obtained from Sigma (USA) and both were used as received without further purification. Single walled carbon nanotubes (SWNTs) of purity > 95% was purchased from Bucky, USA. Phosphate buffer solution (PBS) of pH 7.2 was used as supporting electrolyte. Phosphate buffers of desired pH and ionic strength were prepared according to the method of Christian and Purdy [53] using analytical grade chemicals (NaH_2PO_4 and Na_2HPO_4) from Merck. Stock solutions of adenosine and adenine were prepared in double distilled water and freshly prepared solutions were used each day. All other solvents and chemicals used were of analytical grade.

2.2 APPARATUS AND PROCEDURE

The square wave voltammetric experiments were carried out using a three electrode single compartment cell equipped with single walled carbon nanotubes modified edge plane pyrolytic graphite electrode (SWNT/EPPGE) or bare edge plane pyrolytic graphite electrode (EPPGE) as working electrode, platinum wire as counter electrode and an Ag/AgCl (3M NaCl) as reference electrode (BAS Model MF-2052 RB-5B). The pyrolytic graphite electrode (~ 6 mm^2) used as working electrode for the electro oxidation studies was prepared in the laboratory. Experiments were performed using voltammetric analyzer (Bioanalytical Systems, West Lafayette, IN, USA) Epsilon EC-USB. All the potentials quoted are versus Ag/AgCl electrode at an ambient temperature of 25 ± 2°C.

The pH measurements were carried out using Century India Ltd. Digital pH-meter (Model CP-901) after standardization. Optimized square wave voltammetric parameters used were: initial E: 0 mV, final E: 1600 mV, square wave amplitude (Esw): 25 mV, potential step (E): 4 mV, square wave frequency (*f*): 15 Hz. Working solutions of adenosine and adenine were prepared by adding required volumes of the respective stock solution to the phosphate buffer solution and then voltammograms were recorded. The urine samples of three healthy volunteers were collected and utilized for spiking of standards after 10 times dilution.

2.3 PYROLYTIC GRAPHITE ELECTRODE

Pyrolytic graphite electrode is most versatile electrode used due to its large potential window, low electrical resistance, easily reproducible surface and small residual currents observed in the standard buffer systems. Pyrolytic graphite is prepared by the deposition of pyrolysis of carbon bearing gas under reduced pressure at temperature ranging from 1900-2500 °C. It is anisotropic in nature.

The plane which is perpendicular to the principal axis in a tetragonal or hexagonal structure is called basal plane. Due to nature of the chemical bonding in graphite, the two planes edge and basal, as shown in Fig.1, exhibit completely different electrochemical properties. For electrochemistry the edge plane exhibits considerably faster electrode kinetics in comparison to the basal plane. A FE-SEM images of basal and edge plane are presented in Fig. 2.

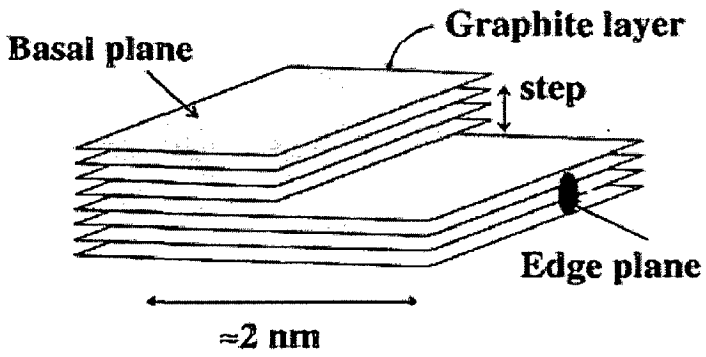
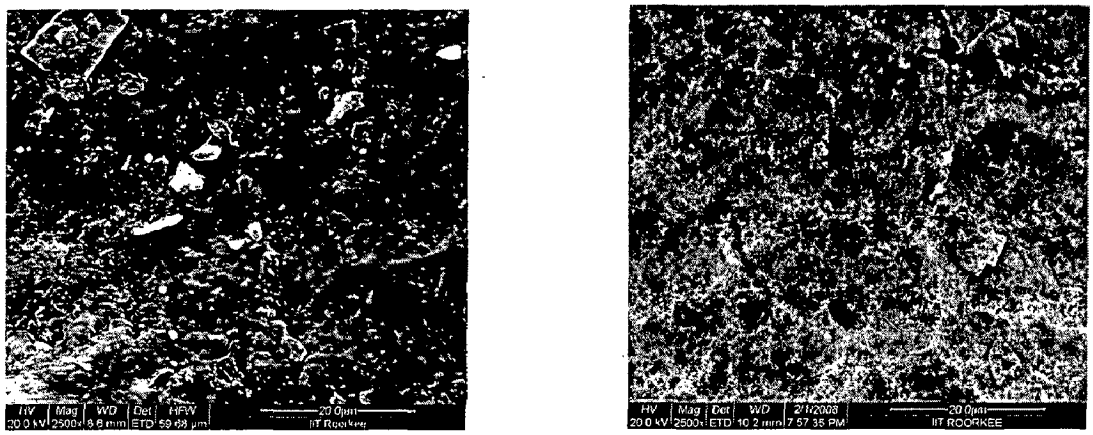


Fig. 1 A typical representation of edge and basal plane in pyrolytic graphite



(A) **(B)**

Fig. 2 A comparison of SEM images of basal plane (A) and edge plane (B) of pyrolytic graphite electrode

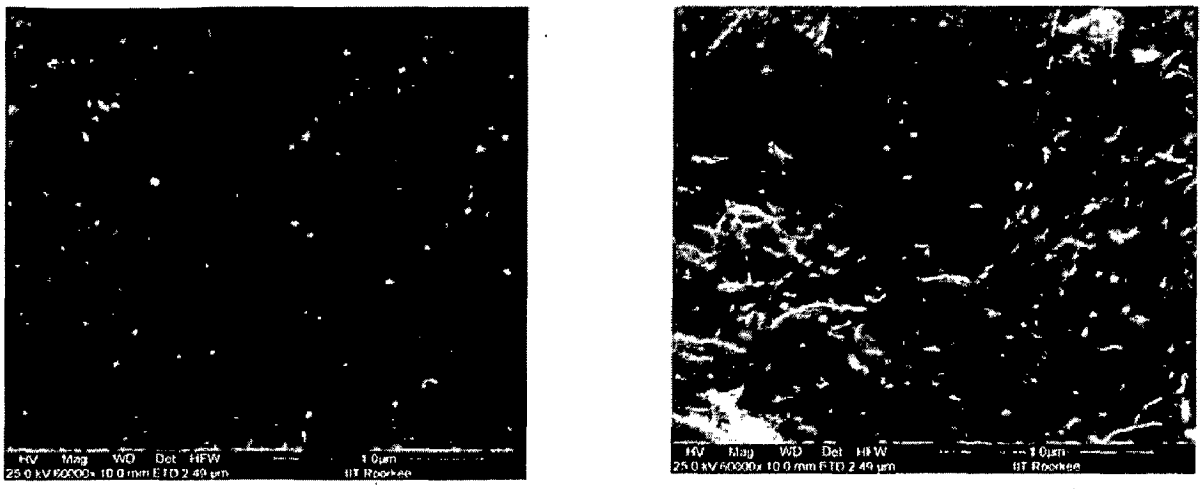
The electrode was prepared in the laboratory by following the procedure as described below:

A pyrex glass tube of appropriate length and diameter was cleaned thoroughly and dried. One end of the glass tube is filled with epoxy resin (Araldite, Ciba Geigy) up to a height of about 2 cm, with the help of a thin gloss rod. Pyrolytic graphite piece was then inserted carefully into the tube from the other end and pushed with the help of a wire so that $3/4^{th}$ portion of the graphite is covered with resin. This procedure prevents the

occurrence of any air pocket between the glass tube and pyrolytic graphite piece. The electrode was then allowed to stand for 24 h until resin solidified. The glass tube was rubbed on a sand paper till the graphite appeared at the resin end. Finally, the electrode was washed several times with distilled water so as to remove the fine powder adhered to the electrode surface of PGE. Mercury was filled into the glass tube and a copper wire was inserted to make proper contact of electrode to the outer circuit. The surface area of PGE was 6 mm^2. The electrode surface was then cleaned by rubbing it on a sand paper, followed by washing with distilled water before recording each voltammogram. As surface area available each time changed due to rubbing, the peak current values observed showed a variation of ± 10%. Hence, an average of three runs was taken for the determinations of peak current values.

2.4 PREPARATION OF THE MODIFIED ELECTRODE

Prior to modification the surface of EPPGE was rubbed on an emery paper followed by cleaning it with double-distilled water and softly touching with tissue paper. Firstly, different concentrations of nanotubes in N, N-dimethyl formamide (DMF) were prepared. Then, 0.5 mg/ml was selected as optimum based on the optimum current response of fixed concentration of adenine or adenosine. A 0.5 mg/ml suspension of SWNT was prepared by dispersing 0.5 mg SWNT in 1.0 ml DMF by ultrasonic agitation. A known volume (40 μL) of this suspension was coated onto the surface of the bare EPPGE and the solvent was allowed to evaporate at room temperature. The modified electrode was now ready for use. The surface morphology of the bare and modified electrode was characterized by recording FE-SEM using Quanta 200 FE-SEM instrument. A comparison of FE-SEM images of the bare and SWNT-modified electrodes is presented in Fig. 3 and clearly indicates the deposition of SWNTs on the electrode surface.



(A) (B)

Fig. 3 A comparison of FE-SEM images of bare (A) and SWNT modified (B) pyrolytic graphite

2.5 EFFECT OF THE AMOUNT OF SWNTs

The effect of different quantity of SWNTs on the peak current response of adenosine and adenine was studied. Different volumes of the suspension prepared by dispersing SWNTs in *N, N* dimethylformamide were casted on the electrode surface. It was observed that up to 40 µL an increase was found in the peak current of fixed concentration of adenine or adenosine. The peak current of both the compounds became almost constant when SWNTs volume was increased more than 40 µL. Therefore, it can be concluded that 40 µL is the optimum amount of SWNTs required for catalyzing the oxidation of adenine and adenosine.

2.6 SURFACE AREA

The effective surface areas of bare and SWNT/EPPGE were obtained by recording cyclic voltammograms of 1 mM $K_3Fe(CN)_6$ containing 0.1 M KCl at various scan rates. A well-defined redox couple was observed due to Fe^{+3}/Fe^{+2} at both electrodes having peak-to-peak potentials separation was larger at bare EPPGE (~ 83.3 mV) than that on

SWNT/EPPGE (~ 61 mV) and peak current was also high at modified electrode. A typical set of cyclic voltammograms observed for ferro-ferricyanide couple is presented in Fig 4. The corresponding reversible processes follow the equation:

$$i_p = 0.4463\,(F^3/RT)^{1/2}\,An^{3/2}\,D_R^{1/2}\,C_0\,v^{1/2}$$

where F is the Faraday's constant (96,485 C/mol), R is the universal gas constant (8.314 J/mol K), A is the surface area of electrode (cm^2), i_p refers to the peak current (μA), n = 1 for $K_3Fe(CN)_6$, T is the absolute temperature (298 K), $D_R = 7.6 \times 10^{-6}$ cm^2s^{-1}, v is the scan rate ($V\ s^{-1}$) and C_0 is the concentration of $K_3Fe(CN)_6$ in mol L^{-1}. The surface areas of bare and modified electrodes were calculated from the slope of the i_p versus $v^{1/2}$ plot and found as 0.0456 and 0.1140 cm^2 respectively. The effective working area of modified electrode is 2.5 times larger than that of bare EPPGE. This indicates that among other reasons higher surface area also may be one of the reasons for improved electrocatalytic activity of modified electrode.

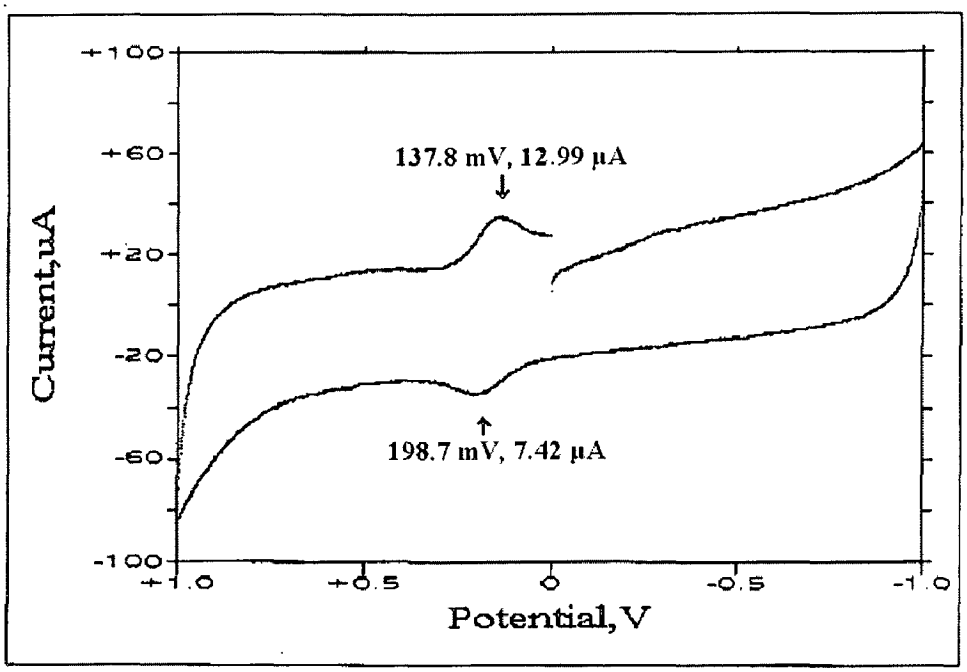


Fig. 4(a) Cyclic voltammogram of $K_3Fe(CN)_6$ using SWNT/EPPGE at scan rate 100 mV/sec.

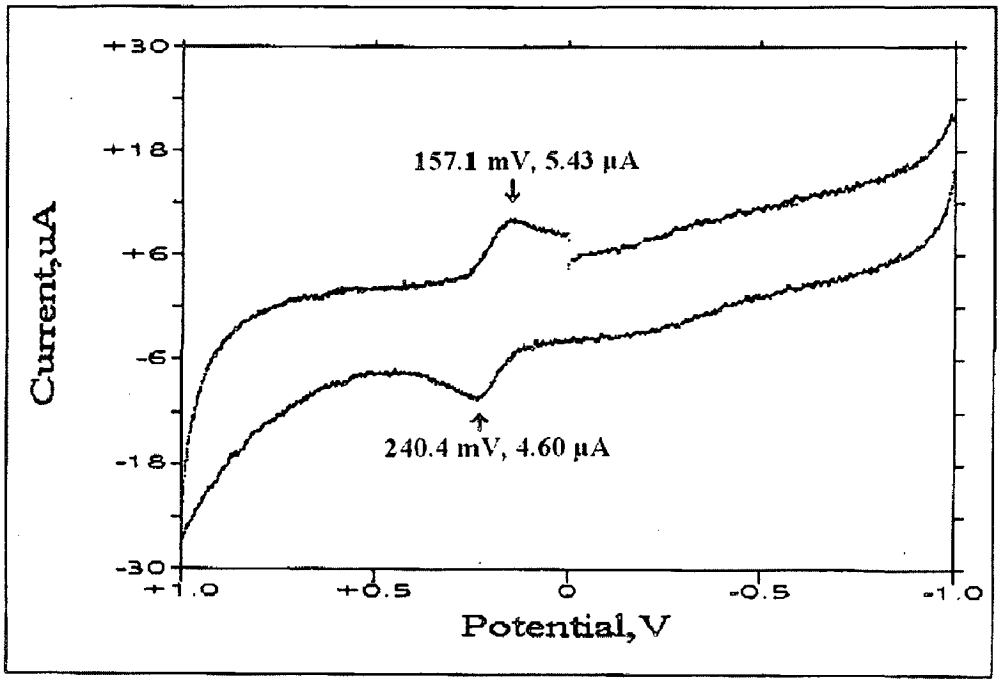


Fig. 4(b) Cyclic voltammogram of $K_3Fe(CN)_6$ using bare EPPGE at scan rate 100 mV/sec.

2.7 METHODOLOGY

Cyclic voltammetry is often the first experiment performed in an electroanalytical study of a compound or biological material at an electrode surface. The effectiveness of cyclic voltammetry results from its ability to rapidly provide considerable information about the thermodynamics of redox process, kinetics of heterogeneous electron transfer reactions, coupled chemical reactions and adsorption processes. Cyclic voltammetry consists of scanning linearly the potential of a stationary working electrode (in an unstirred solution) using a triangular potential waveform as shown in Fig. 5. Single or multiple cycles can be used depending on the information sought. During the potential sweep, the potentiostat measures the current resulting from the applied potential. The resulting plot of current versus potential is termed a *cyclic voltammogram*. The cyclic voltammogram is a time dependent function of a large number of physical and chemical parameters.

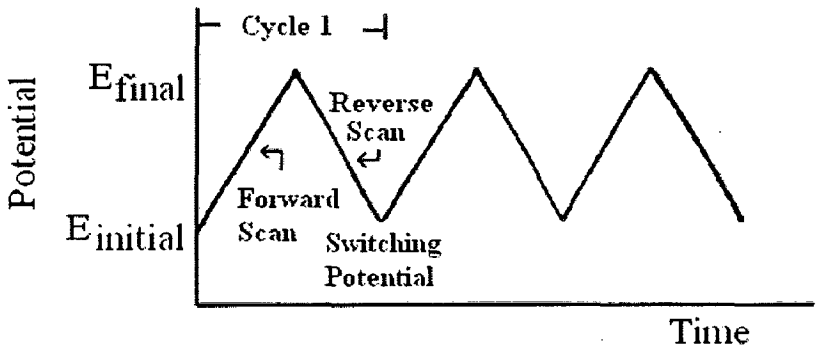


Fig. 5 Potential-time excitation signals in cyclic voltammetric experiment

The peak current for a reversible couple (at 25°C), is given by the *Randles-Sevcik equation*:

$$i_p = (2.69 \times 10^5)\ n^{3/2}\ \mathrm{ACD}^{1/2}\ v^{1/2}$$

where i_p is peak current in ampere, n is the number of electrons, A is the electrode area (cm^2), C is the concentration (in mol/cm^3), D is the diffusion coefficient (in cm^2/s) and v is the scan rate (in V/s). Accordingly, the current is directly proportional to concentration and increases with the square root of the scan rate. The ratio of the reverse-to-forward peak currents is unity for a simple reversible couple.

Square wave voltammetry is a large-amplitude differential technique in which a wave form consists of a square wave of constant amplitude superimposed on a base staircase potential. The current is sampled twice during each square wave cycle, once at the end of the forward pulse (at t_1) and once at the end of the reverse pulse (at t_2). This difference current ($i_f - i_r$) is plotted as a function of base staircase potential. There are two advantages to measuring the difference current. First, it increases the discrimination against the charging current, since any residual charging current is subtracted out. Second, the shape of the current response is a symmetric peak rather than the sigmoidal curve typically found for normal pulse voltammetry. Coupled with the effective discrimination against the charging background current very low detection limit near 1×10^{-8} M can be attained.

Chapter-3

RESULTS AND DISCUSSION

3.1 COMPARISON OF BARE AND MODIFIED ELECTRODE

It is prime step to choose appropriate substrate for voltammetric experiments hence; firstly voltammograms were recorded using both bare and modified electrodes in order to find which one is better towards the simultaneous oxidation of adenine and adenosine. Electrochemical properties of adenosine and adenine were demonstrated by using square wave voltammetry at bare EPPGE and SWNT/ EPPGE in phosphate buffer solution of pH 7.2. Fig. 6 clearly indicates that on scanning the potential from 0 to 1600 mV two oxidation peaks were noticed at ~ 928 mV and ~1230 mV using the bare electrode while for the modified electrode oxidation peak were appeared at ~856 mV and ~1190 mV having marked increment in current value as compared to the bare electrode for adenine and adenosine, respectively. The significant improvement in peak current with decreasing potential at modified electrode clearly demonstrate that single walled carbon nanotubes act as an efficient electron mediator for oxidation of adenine and adenosine. The higher surface area of modified electrode, edge-plane-like defects which are present at the open ends of nanotubes and embedded metal impurities in CNT samples are some important reasons responsible for electrocatalytic properties of nanotubes [54, 55]. The SWNT/EPPGE acts as better substrate for the voltammetric oxidation of both compounds therefore, further detailed studies were carried out using nanotubes modified electrode.

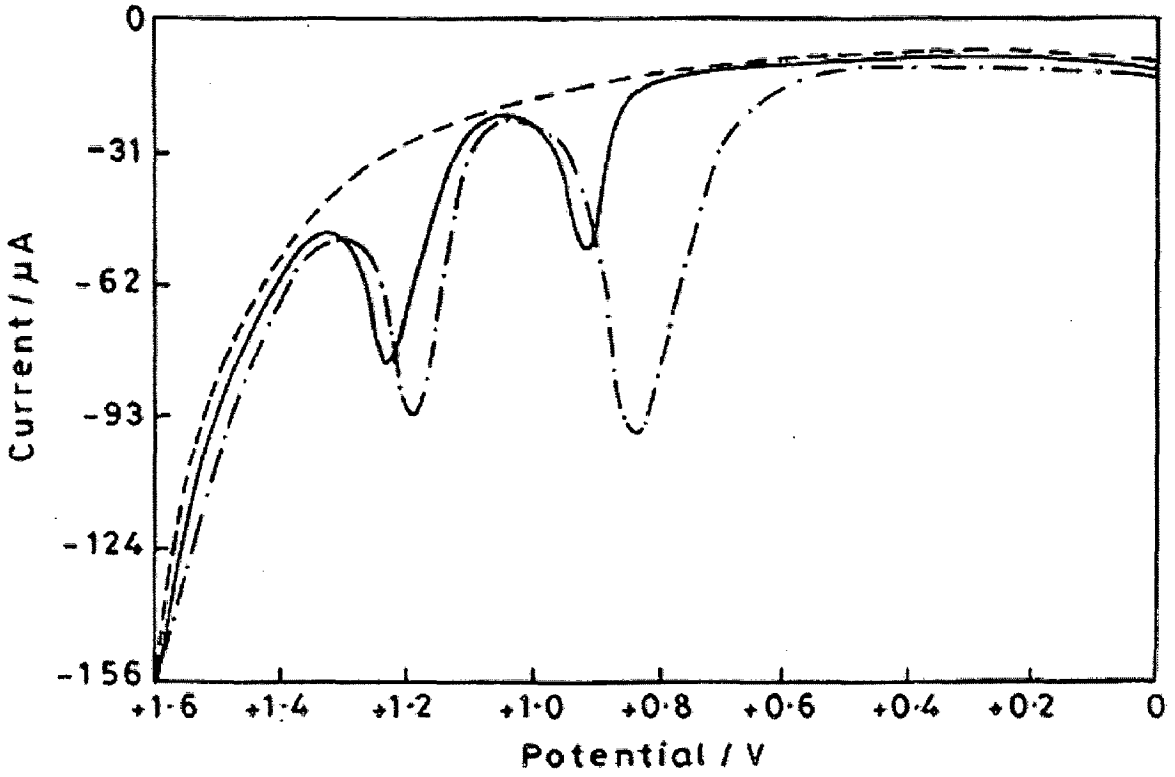


Fig. 6 Observed square wave voltammograms for the oxidation of 50 μM of each adenine and adenosine at bare EPPGE (——), SWCNT modified EPPGE (-·-·-·) and background PBS (pH 7.2) at SWNT modified EPPGE (------).

3.2 CYCLIC VOLTAMMETRY

Electrochemical response of a solution having 50 μM of adenine was estimated by recording cyclic voltammogram at 50 mV/s after bubbling N_2 for 10 min. Anodic peak obtained for the oxidation of adenine was observed at ~ 928 mV as shown in Fig 7. Absence of reduction peak on reverse sweep clearly indicates that adenine is irreversibly oxidized at SWNT modified EPPGE.

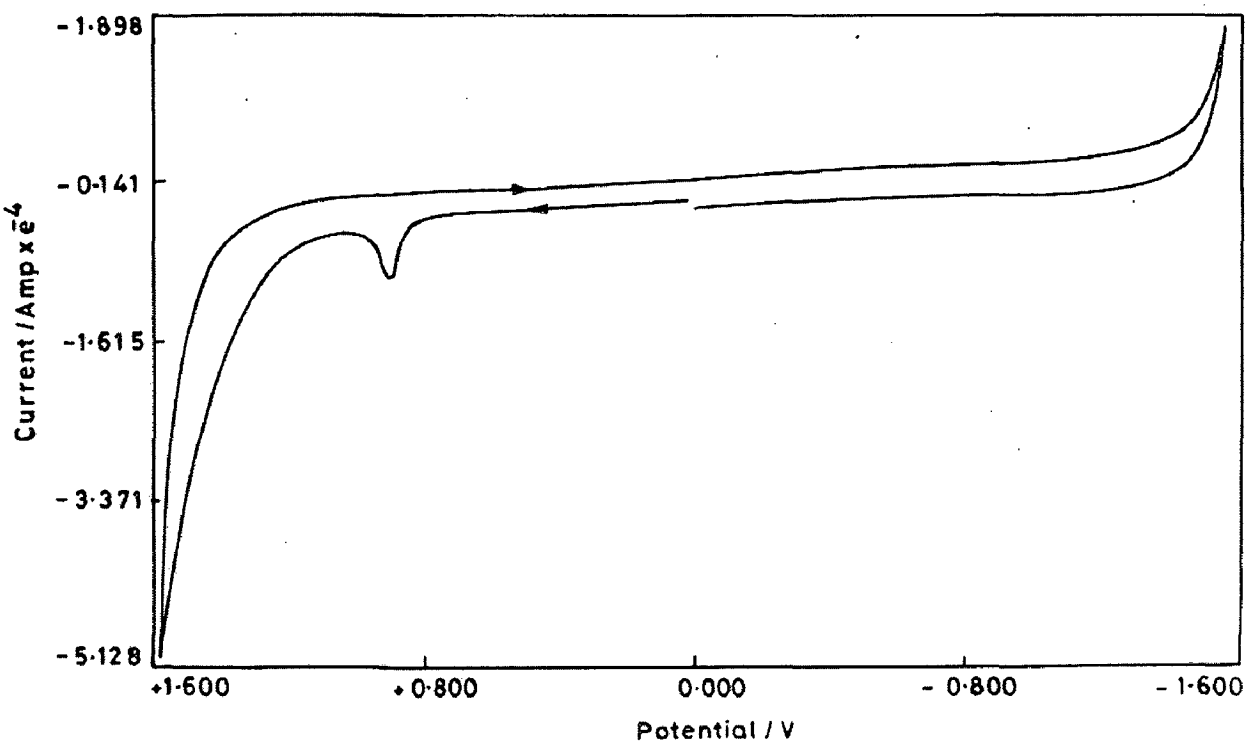


Fig. 7. A typical cyclic voltammogram observed for 50 μM adenine at SWNT/EPPGE

In order to determine nature of electrode reaction towards adenosine, cyclic voltammogram of blank phosphate buffer solution was recorded using SWNT/EPPGE after N_2 purging for 10 min. and then cyclic voltammogram of a solution having 50 μM adenosine was recorded under optimized parameters. Only anodic peak was obtained for the oxidation of adenosine at ~1230 mV as shown in Fig 8.

Absence of any reduction peak in the reverse sweep clearly indicates that the electrode reaction is irreversible in nature.

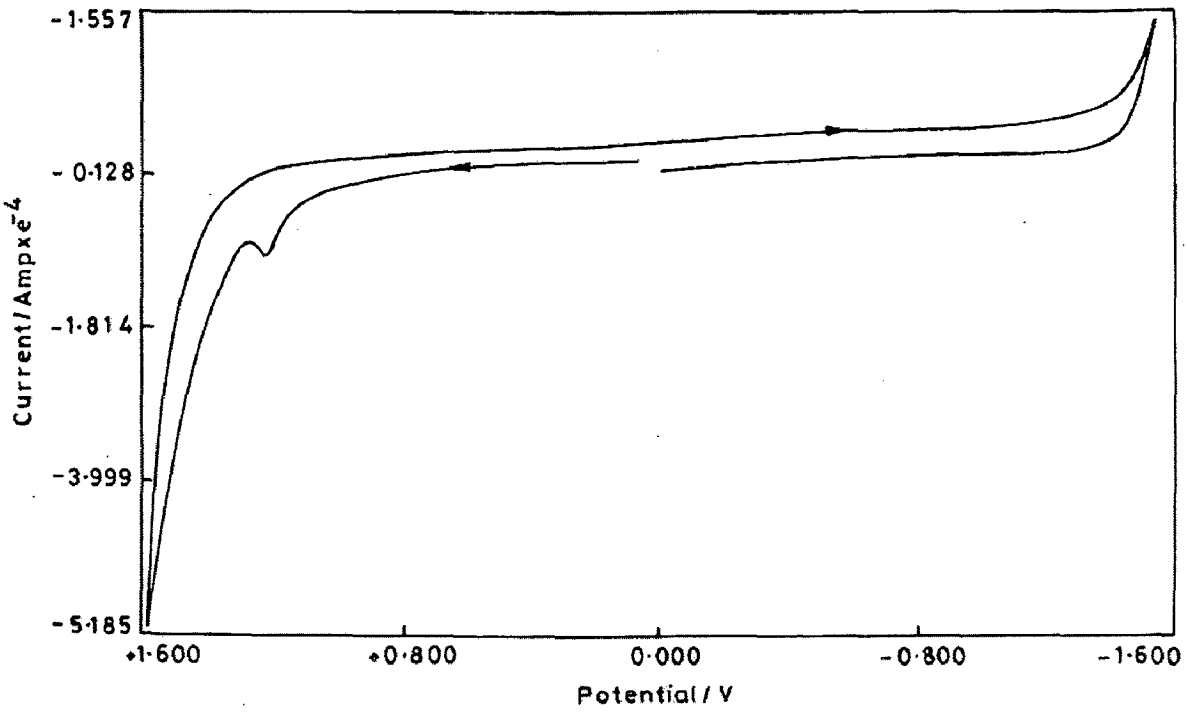


Fig. 8 Cyclic voltammogram observed for 50 μM adenosine at pH 7.2 using SWNT/EPPGE

Fig. 9 presents a cyclic voltammogram of 50 μM of each adenine and adenosine in phosphate buffer of pH 7.2 at SWNTs modified edge plane pyrolytic graphite electrode. Firstly, cyclic voltammogram was recorded in a blank PBS of pH 7.2 and then binary solution of adenine and adenosine was examined. Two well defined peaks for the oxidation of adenine and adenosine were observed at potentials ~ 928 mV and ~ 1230 mV, respectively at SWNT/EPPGE. The peaks obtained for both the compounds were in the form of broad bulges at bare EPPGE suggesting thereby slow electron transfer kinetics at bare electrode hence, the attempts to use bare EPPGE for the determination of both compounds failed by cyclic voltammetry. At the SWNT modified electrode well-defined oxidation peaks were obtained and the peak current was also found to increase. The above results suggested that the SWNT modified electrode promoted the electrochemical

oxidation of adenine and adenosine by considerably accelerating the rate of electron transfer. Square wave voltammetry is more sensitive technique with well established advantages such as discrimination against background current, low detection limit and high sensitivity as compared to cyclic voltammetry hence, further detailed studies were carried out using square wave voltammetry.

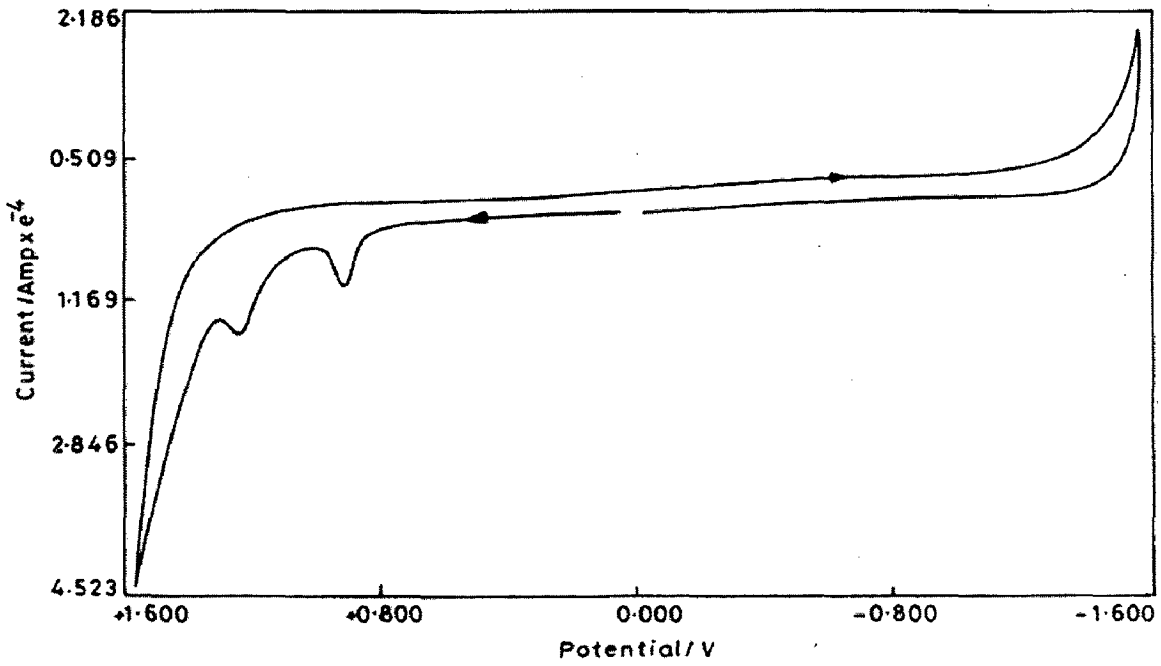


Fig. 9 Cyclic voltammogram for the oxidation of a binary solution containing 50 μM of each adenine and adenosine at SWCNT/EPPGE

3.3 SQUARE WAVE VOLTAMMETRY

3.3.1 Electrochemical Behavior of Adenine

The pH of supporting electrolyte is an important parameter that affects redox behavior of biomolecules and drugs. The effect of pH on oxidation peak potential of adenine was evaluated in the pH range of 2.37 – 10.97. It was observed that peak potential of adenine shifted to less positive potential with increase in pH as shown in Fig 10 (a). The

E_p vs. pH plot was linear and dependence of anodic peak potential of the analyte on the pH of supporting electrolyte can be presented by the following equation:

$$E_p \text{ /mV } [2.37 - 10.97] = -58.73 \text{ pH} + 1365.6 \text{ versus Ag /AgCl}$$

having correlation coefficients 0.991. The value of $dE_p / dpH \sim 59$ mV/pH indicates the involvement of equal number of protons and electrons ($6e^-$, $6H^+$) in the oxidation reaction of adenine. As shown in reaction mechanism (Scheme I), an unstable diimine is formed through primary electrode reaction which on hydrolysis leads to number of products [56].

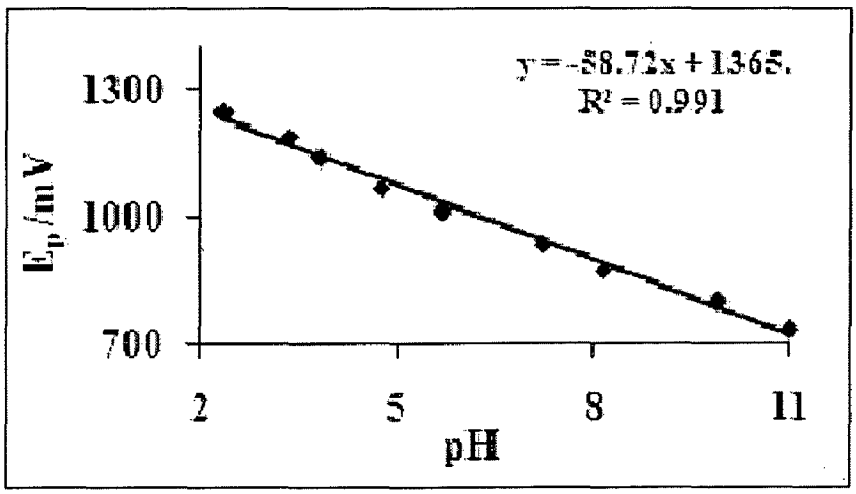
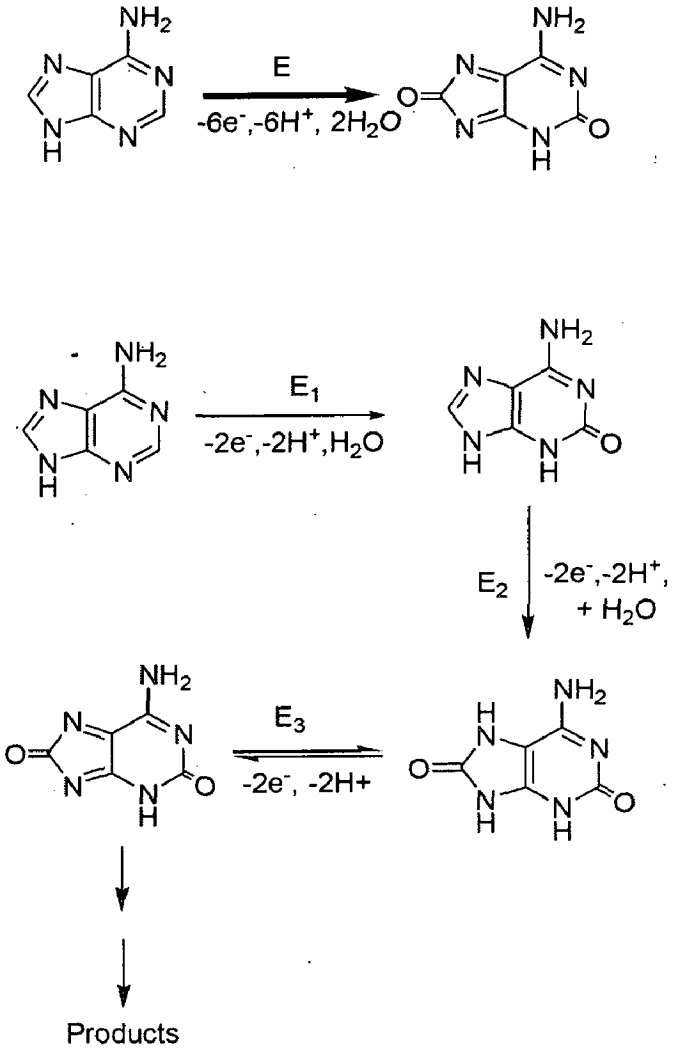


Fig. 10(a). The observed dependence of E_p on pH for adenine



Scheme I: Oxidation reaction mechanism for adenine

The variation of square wave frequency (*f*) with peak current (i_p) of adenine was studied in the frequency range of 5–45 Hz at pH 7.2. The peak current (i_p) of 50 μmol L^{-1} adenine shows a linear increase with square root of square wave frequency as shown in Fig. 10(b) suggesting thereby that electrode reaction for the oxidation of adenine is diffusion controlled process. Linear relations between i_p and $f^{1/2}$ for adenine can be expressed by the following equation:

$$i_p\ /\mu A = 3.284\, f^{1/2}\ (\mathrm{Hz}) - 6.191$$

with correlation coefficient of 0.989.

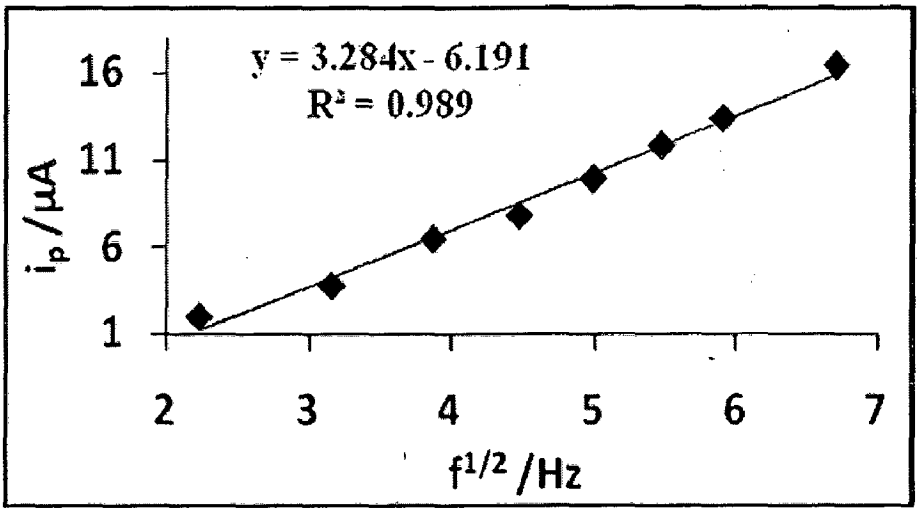


Fig. 10(b) The observed plot of peak current versus $f^{1/2}$ for adenine at pH 7.2

It is well known that peak current depends on the concentration of analytes hence, square wave voltammograms were recorded for various concentration of adenine in phosphate buffer solutions of pH 7.2 as shown in Fig. 10(c). It was observed that with increasing the concentration of adenine, oxidation peak current was found to increase linearly. The oxidation peak current versus concentration plots showed a good linearity for adenine in the concentration range of 5– 80 μmol L^{-1} as depicted in inset of Fig. 10(c). Current values were obtained by subtracting the background current of buffer solution and average of three replicate measurements was used to plot calibration curve. Linear regression equation obtained from calibration plot can be represented as:

$$i_p\ (\mu A) = 0.467\ C\ (\mu mol\ L^{-1}) + 1.168 \text{ versus Ag/AgCl}$$

with a correlation coefficients of 0.988. The limit of detection was calculated by using the formula $3\sigma/b$, where σ is the standard deviation of blank solution and b is the slope of calibration curve. The detection limit and sensitivity were found to be 1.1 μM and 0.467 μA/μM, respectively.

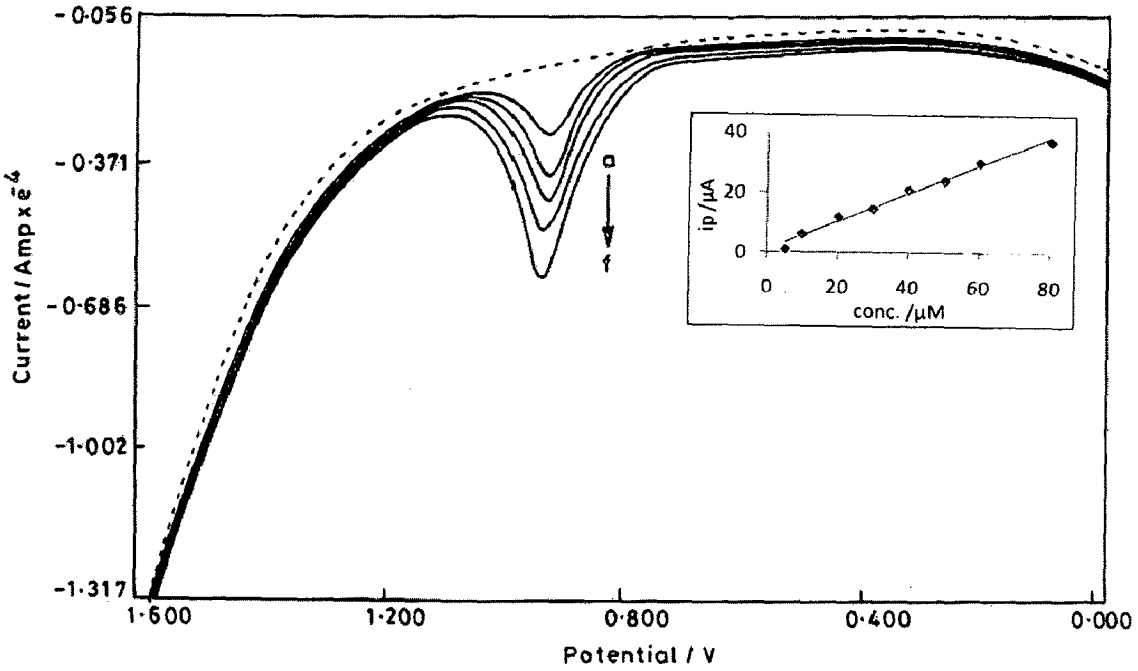


Fig. 10(c) Square wave voltammograms recorded for different concentrations ranging from 5– 80 μM of adenine, inset shows the calibration plot for adenine.

Mechanism of Adenine oxidation

The oxidation chemistry of adenine has been studied by many workers at pyrolytic graphite and glassy carbon electrodes. The 6e, $6H^+$ process has been found to give diimine species, which on follow up chemical steps give pH dependent products. The 6e, $6H^+$ step can be written as a combination of three 2e, $2H^+$ steps as shown above. The first 2e, $2H^+$ step leads to the formation of 2-hydroxyadenine, which on further oxidation gives 2,8-dihydroxyadenine. The possibility of the formation of 8-hydroxyadenine in the first 2e, $2H^+$ oxidation step has been ruled in the literature using CV studies. In the final step the 2e, $2H^+$ oxidation leads to the formation of diimine species. As C=N bonds are suceptible to hydrolysis, the rapid hydrolysis gives pH dependent products [56].

3.3.2 Electrochemical Behavior of Adenosine

The electro oxidation of adenosine was carried out by square wave voltammetry at the surface of SWNT modified EPPGE over the pH range 2.37 to 10.97. It was observed that peak potential of adenosine shifted to less positive potential with increase in pH as shown in Fig 11 (a).

The E_p vs. pH plot is linear and dependence of anodic peak potential of the analyte on the pH of supporting electrolyte can be presented by the following equation:

$$E_p / mV \ [2.37 - 10.97] = \ -53.78 \text{ pH} + 1614 \text{ versus Ag /AgCl}$$

having correlation coefficients 0.997. The value of $dE_p / dpH \sim 54 \ mV / pH$ indicates the involvement of equal number of protons and electrons ($6e^-$, $6H^+$) in the oxidation reaction of adenosine as shown in oxidation reaction mechanism (Scheme II) of adenosine [57].

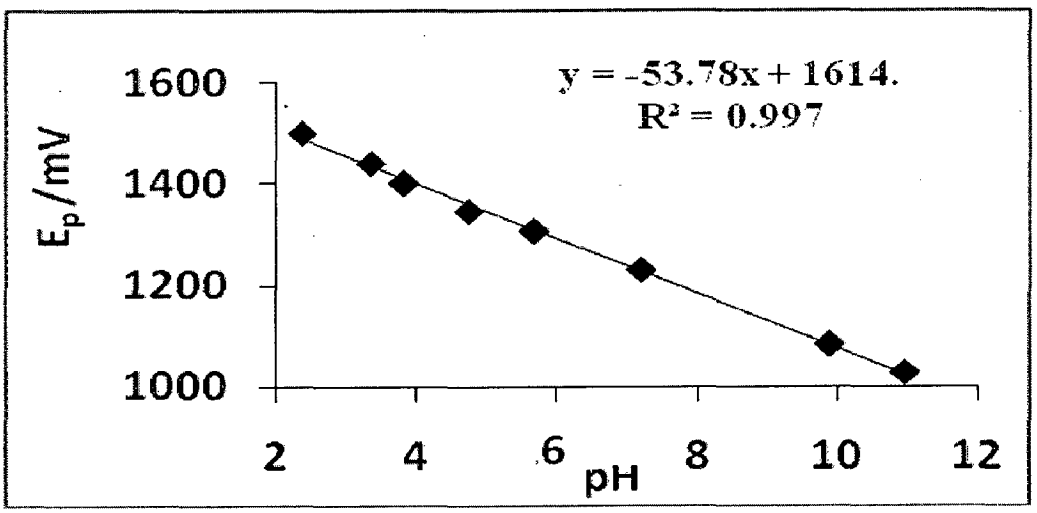
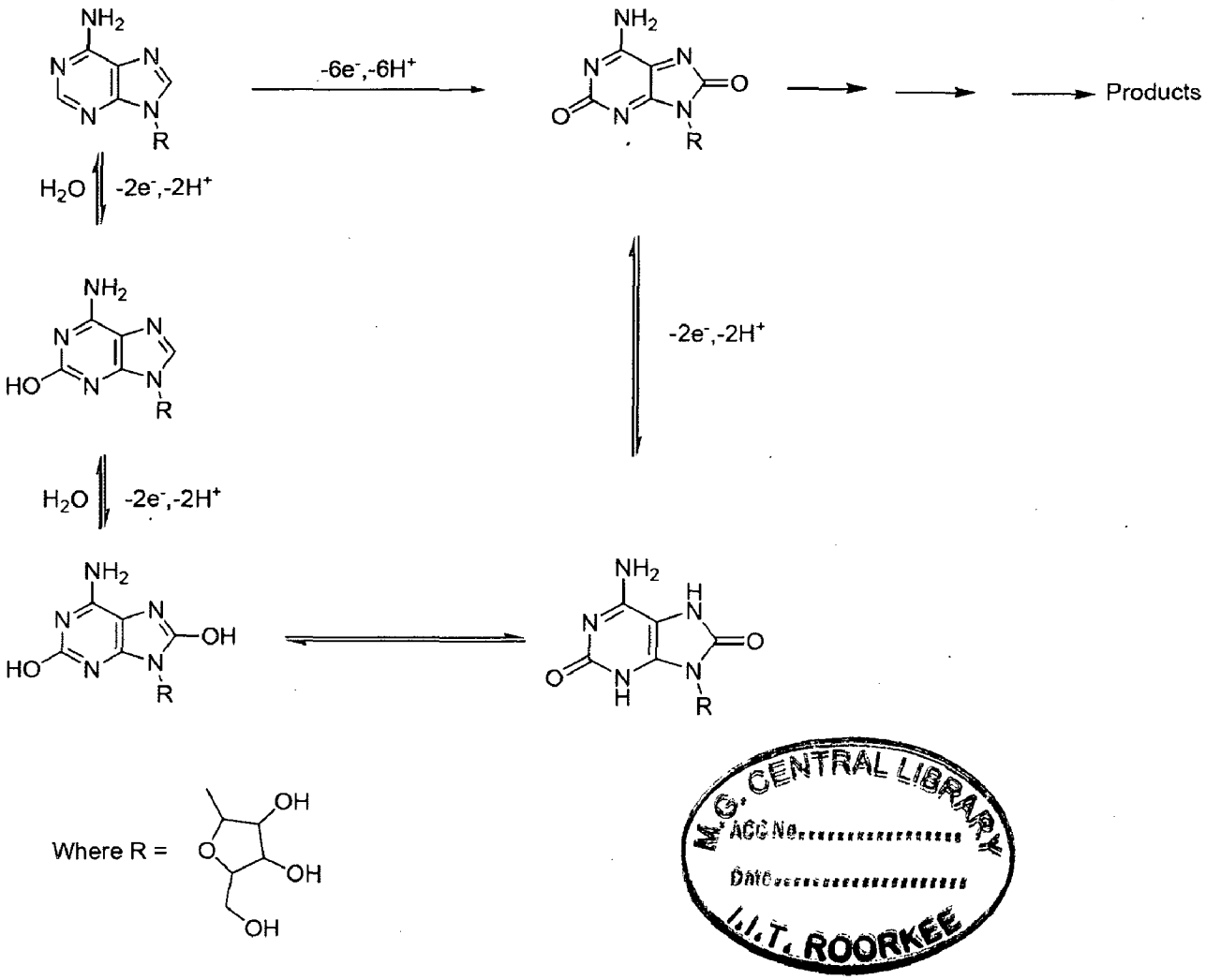


Fig. 11 (a) The observed dependence of E_p on pH for adenosine



Scheme II: Oxidation reaction mechanism for adenosine

The influence of square wave frequency (*f*) on peak current of adenosine was examined in the frequency range 5–35 Hz at pH 7.2 using SWNT/EPPGE. The peak current of 50 μmol L^{-1} adenosine shows a linear increase with square root of square wave frequency as shown in Fig. 11(b) suggesting thereby that electrode reaction for oxidation of adenosine is diffusion controlled process. Linear relations between i_p and $f^{1/2}$ for adenosine can be expressed by the following equation:

$$i_p / \mu A = 5.563 f^{1/2} (Hz) - 8.266$$

with correlation coefficient of 0.975.

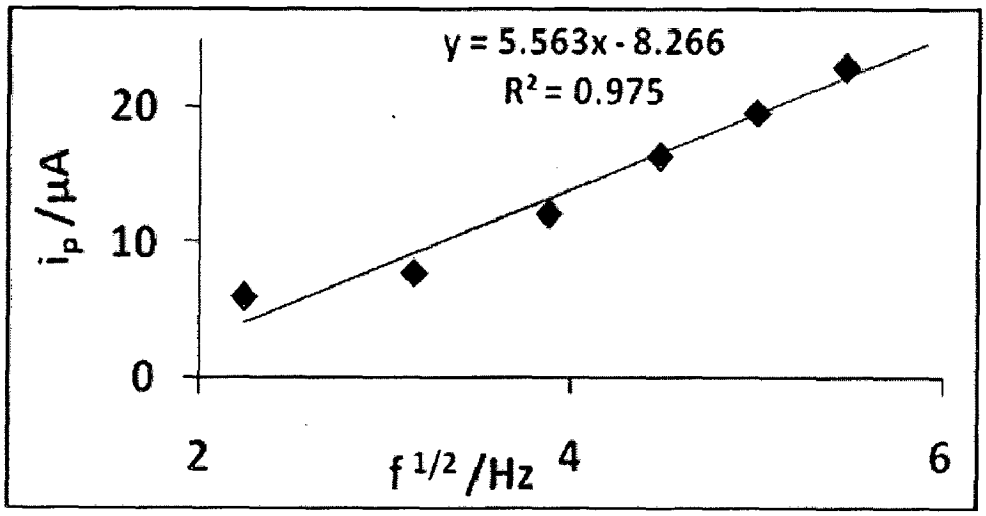


Fig. 11(b) The plot of peak current vs. $f^{1/2}$ for adenosine at pH 7.2

Square wave voltammograms were recorded for the various concentrations of adenosine in phosphate buffer solutions of pH 7.2 as shown in Fig. 10(c). It was observed that with increasing the concentration of adenosine, oxidation peak current was found to increase linearly. The oxidation peak current versus concentration plot showed a good linearity for adenosine in the concentration range of 10 – 90 μmol L^{-1} as depicted in inset of Fig. 11 (c). Linear regression equation arises from calibration plot can be expressed as:

$$i_p\ (\mu A) = 0.377\ C\ (\mu mol\ L^{-1}) + 0.575 \text{ versus Ag/AgCl}$$

with a correlation coefficients of 0.992. The limit of detection was calculated by using the formula $3\sigma/b$, where σ is the standard deviation of blank solution and b is the slope of calibration curves. The detection limit and sensitivity were found to be 2.9 μM and 0.377 μA/μM, respectively.

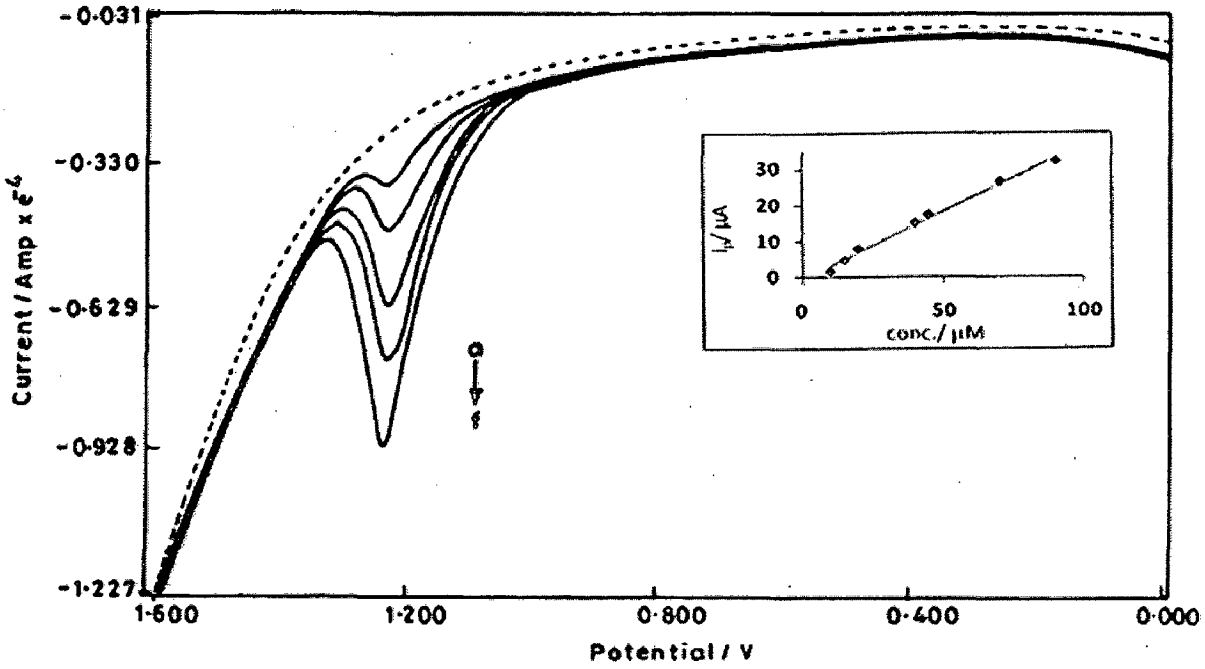


Fig. 11(c) Square wave voltammograms recorded for different concentrations of adenosine ranging from of 10 – 90 μM, inset shows the calibration plot for adenosine.

3.3.3 Simultaneous Determination of Adenine and Adenosine

Square wave voltammograms were recorded to evaluate the electrochemical response of different concentrations of adenosine and adenine when both the substances are present in the same solution. For this purpose firstly the concentration of adenine was kept constant at 20 μmol L^{-1} while concentration of adenosine was varied from 10 to 80 μmol L^{-1} as shown in Fig 12. Analogously, as shown in Fig. 13 the influence of adenine concentration was checked by increasing the adenine concentration from 10 to 100 μmol L^{-1} while fixing the adenosine concentration constant at 30 μmol L^{-1}. Examination of the obtained results indicate that the oxidation peak current for adenosine systematically increases as its concentration increased at a fixed concentration of adenine (whose oxidation peak current remains fairly constant). Similarly oxidation peak current for adenine systematically increases as its concentration increased at a constant concentration

of adenosine (whose oxidation peak current remains constant). Square wave voltammograms with different concentrations of both adenosine and adenine in the same solution were also recorded and it was found that when concentrations of both compounds increase simultaneously, both compounds exhibits oxidation peaks separately without interfering each other. It was also found that oxidation peaks observed for adenosine and adenine in same solution not only do not interfere with each other using nanotubes modified edge plane pyrolytic graphite electrode but also the peak current values are exactly similar to those obtained from individual calibration plots of adenosine and adenine. Thus, it can be concluded that the proposed sensor can be successfully applied for the simultaneous determination of adenosine and adenine in real samples.

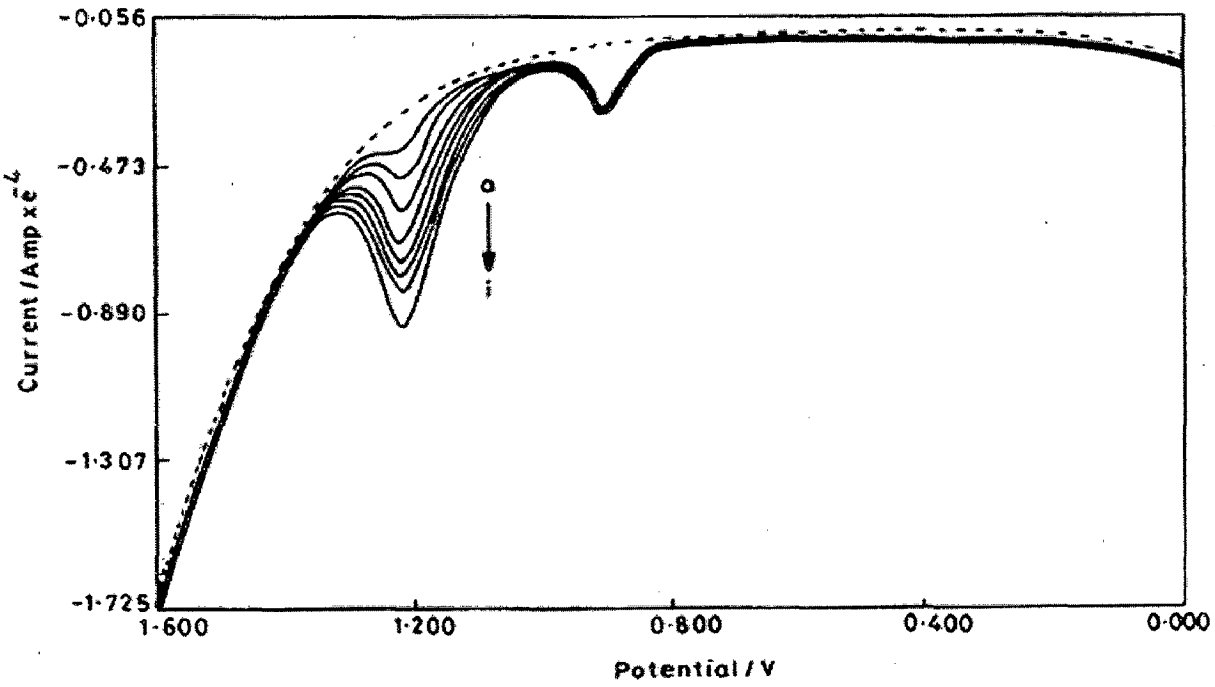


Fig. 12 Square wave voltammograms recorded for different concentrations of adenosine keeping the adenine concentration constant.

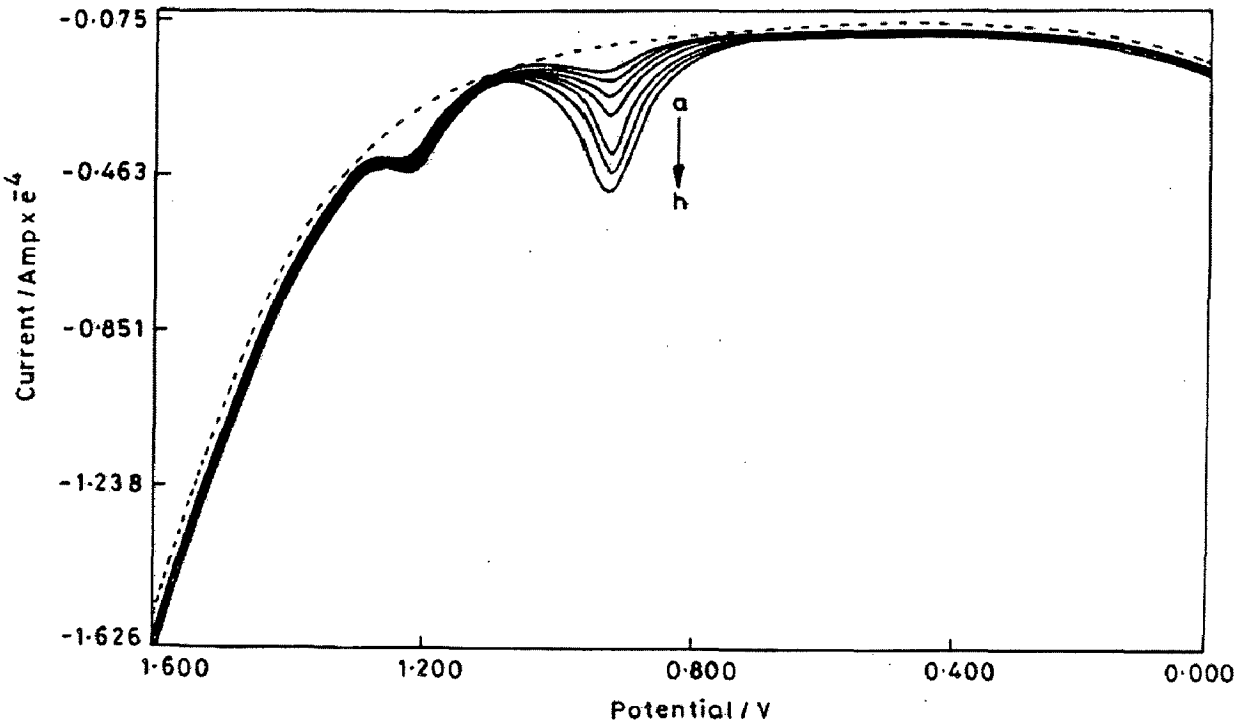


Fig. 13 Square wave voltammograms recorded for different concentrations of adenine keeping the adenosine concentration constant.

3.4 ANALYTICAL APPLICATIONS

In order to examine the practical utility of the proposed method recovery experiments were performed using standard addition method. Three human urine samples obtained from healthy volunteers were spiked with known amounts of standard adenine and adenosine ranging from 10 μM to 50 μM subsequently followed by recording their voltammograms utilizing SWNT modified EPPGE. In all the cases two separate well- defined peaks were observed with E_p ~ 856 and ~ 1190 mV corresponding to the oxidation of adenine and adenosine, respectively. The concentration of the two compounds was calculated using calibration plots and the results observed are listed in Table 1. The recoveries varied in the range from 96.80 % to 101.60 % in the case of adenine and from 97.20 % to 103.60 % in case of adenosine. The recovery values signify the passable accuracy of the proposed method.

Table 1 Recovery results obtained for adenine and adenosine in human urine samples using SWNT/EPPGE

Adenine			Adenosine	
Spiked (μM)	Detected (μM)	Recovery (%)	Detected (μM)	Recovery (%)
Sample 1				
10.0	10.16	101.60	9.89	98.90
30.0	29.84	99.47	30.24	100.80
50.0	50.70	101.40	51.80	103.60
Sample 2				
10.0	9.68	96.80	9.72	97.20
30.0	30.34	101.13	30.96	103.20
50.0	49.50	99.00	50.0	100.00
Sample 3				
10.0	10.00	100.00	9.76	97.60
30.0	30.09	100.30	30.16	100.53
50.0	49.95	99.90	51.08	102.16

3.5 STABILITY AND REPRODUCIBILITY OF THE MODIFIED ELECTRODE

The long term stability and reproducibility of modified electrode are important parameters for analytical purposes. The SWNT/EPPGE was evaluated by measuring the voltammetric current response of fixed concentration of adenosine or adenine (50 μM) after the modified electrode was stored for approximately 1 week. Only a minimal decrease of current sensitivity with a relative standard deviation (R.S.D.) of about 3.82 % for adenosine and 4.45 % for adenine was observed which can be attributed to the excellent stability of the modified electrode. The reproducibility of modified electrode has also been investigated. The intra-day precision of the method was evaluated by repeating six experiments in the same solution containing 50 μM of adenosine and 50 μM of adenine taken separately using the same SWNT/EPPGE. The R.S.D. was found to be 0.74 % and 1.26 % for adenosine and adenine, respectively, indicating excellent reproducibility

of the modified electrode. Further, inter-day precision was investigated by measuring the current response of the modified electrode for 6 consecutive days for the same concentration of adenosine and adenine (50 μM) taken separately and the respective relative standard deviations were found to be 1.37 % and 2.43 %. Thus, it is recommended that SWNT/EPPGE can be used safely for a week with reproducible results.

Chapter-4
CONCLUSION

The results presented above make obvious that adenine and adenosine can be simultaneously determined using square wave voltammetry at SWNT modified edge plane pyrolytic graphite electrode. The modified electrode displayed strong catalytic function towards the oxidation of both compounds which not only shifted oxidation peak potentials towards less positive potentials but also increased the peak currents. Both the nucleosides have also been determined in human urine samples with good sensitivity and reproducibility. The proposed method offers several advantages over the methods described previously for the determination of adenine and adenosine [20, 22, 47] such as fast response, high sensitivity, low detection limit. Moreover, proposed voltammetric method can be used without tedious and time consuming sample preparation, derivatization and extraction steps which are essentially required for conventional methods [20, 22, 47]. Owing to its adequate stability, selectivity and sensitivity the modified electrode could provide a promising tool for the simultaneous determination of adenine and adenosine in complex biological samples. The developed method is fast and cost effective tool for detecting various metabolic disorders and malfunctioning of various systems in human body and a promising substitute to other reported methods.

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