VOLTAMMETRIC SENSORS FOR THE DETERMINATION OF BIOMOLECULES/DOPING AGENTS

A THESIS

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

of

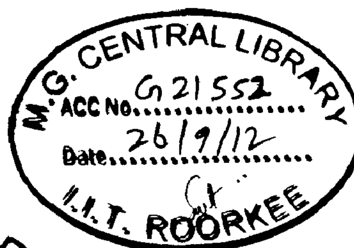
DOCTOR OF PHILOSOPHY

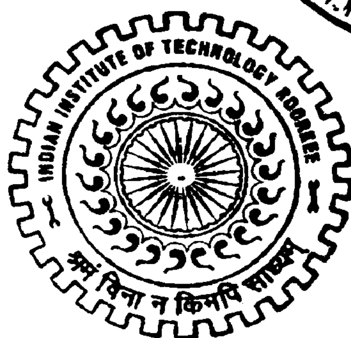
in

CHEMISTRY

by

SUNITA BISHNOI





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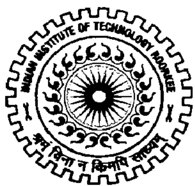

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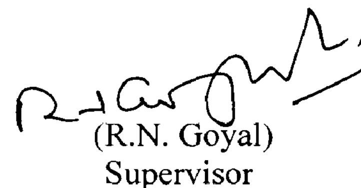
CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled, **"VOLTAMMETRIC SENSORS FOR THE DETERMINATION OF BIOMOLECULES/DOPING AGENTS"** in partial fulfilment of the requirements for the award of the Degree of Doctor of Philosophy and submitted in the Department of Chemistry of the Indian Institute of Technology Roorkee, Roorkee is an authentic record of my own work carried out during a period from July, 2007 to May, 2011 under the supervision of Dr. R.N. Goyal, Professor, Department of Chemistry, Indian Institute of Technology Roorkee, Roorkee.

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

(SUNITA BISHNOI)

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



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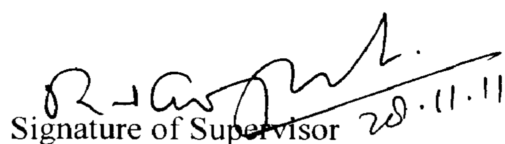
Date: 19-May-2011

The Ph.D. Viva-Voce Examination of **Ms. Sunita Bishnoi,** Research Scholar, has been held on ....28.11.11................



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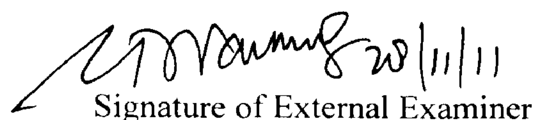

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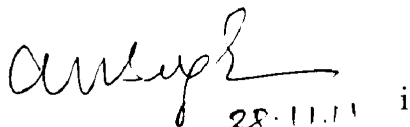
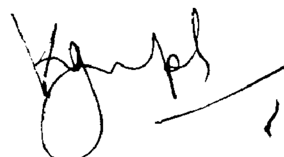




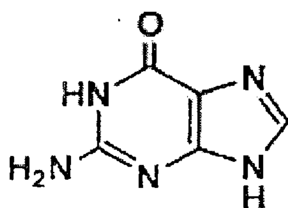
ABSTRACT

The need of highly sensitive and selective sensing probes having enormous practical applications and significant benefits over conventional technologies attracted researchers towards nanomaterials based technologies. Last few years have seen a worldwide outburst of research interest of electrochemists at the interface of nanotechnology and electrochemistry. The implementation of nanoscience and nanotechnology achievements in bioelectrochemistry ameliorates the current electroanalytical techniques and methodologies by improving sensitivity and selectivity along with detection limit. Application of carbon nanomaterials in sensor research offers excellent prospects for designing novel sensing systems and enhancing the performance of bioanalytical assay. The fabrication of electrode surface with nanomaterials imparts functionality distinct from the base electrode and has been found to show consistently good results with high sensitivity and selectivity. In view of the large number of biomolecules in human body and the ability of nanomaterial modified electrodes to measure extremely small amounts of specific biomarkers at molecular levels, an endeavour has been made in the present investigation to develop simple and sensitive electroanalytical methods using nanomaterial based sensors for the qualitative and quantitative analysis of biologically important compounds, drugs and doping agents.

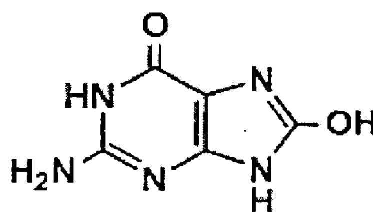
The first chapter of the thesis is "General Introduction" giving a recapitulate review of the relevant work and, highlights the significance of electrochemical studies in biological systems along with its relevance in assorted areas. The brief account of various electroanalytical techniques employed for present dissertation has also been described. The chapter also discusses the concise outline of advantages rendered by nanomaterial modified electrodes over conventional electrodes.

The second chapter of the thesis describes the simultaneous determination of relative concentrations of guanine and 8-hydroxyguanine in oxidatively damaged calf thymus DNA sample. Guanine is highly susceptible to oxidative stress in the genomic DNA due to having the lowest oxidation potential hence; guanine plays a key role in the oxidation of DNA by various types of oxidants and free radicals. 8-Hydroxyguanine is one of the prominent lesions generated during DNA damage by oxidative processes and most extensively investigated due to its miscoding properties and potential role in mutagenesis,

carcinogenesis and aging. A quantitatively important alteration occurring during oxidative damage to DNA consists of oxidation of guanine residues into 8-hydroxyguanine. The bare and single walled carbon nanotubes modified edge plane pyrolytic graphite electrodes are utilized to examine such type of vital alteration. It has been found that remarkable enhancement in the oxidation peak current of both compounds was observed along with the negative shift of peak potentials using modified electrode as compared to bare electrode. Oxidative damage to DNA bases by isotope dilution mass spectrometry required isotopically labelled oxidized bases as internal standards and GC-MS requires a derivatization procedure which can cause 'artifactual' oxidation of some undamaged bases leading to an overestimation of their oxidation products, including 8-hydroxyguanine. Therefore, using proposed method which requires no internal standards and derivatization steps and, hence, eliminates possible 'artifactual' oxidation of DNA bases; the level of guanine and 8-hydroxyguanine can be measured in calf thymus DNA with high rank of accuracy.



Guanine



8-Hydroxyguanine

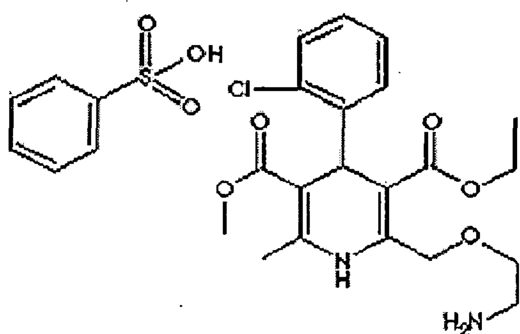
The third chapter of the thesis presents the novel approach using square wave voltammetry with the combination of multi-walled carbon nanotubes modified edge plane pyrolytic graphite electrode (MWNT/EPPGE) as a sensitive and selective sensor for the detection of catecholamines – epinephrine (EP) and norepinephrine (NE) in human body fluids. The chapter is divided into two sections – first section deals with the determination of epinephrine while the second section describes the simultaneous determination of epinephrine and norepinephrine. Catecholamines are produced by sympathetic nervous system activation and act as hormones and neurotransmitter to monitor heart rate, brain muscles activity, glycogenolysis, fatty acid mobilization and body temperature. These important actions also make them potent doping agents and hence, epinephrine is banned in competitive games by World Anti Doping Agency. Studies show that changes of their

concentration in nervous tissues and body fluids are diagnostic symptoms of several diseases. The amount of catecholamines present in blood, plasma or serum is considered as a diagnostic aid to monitor therapeutic administration or to identify the causative agent in potential poisoning victims. Hence, the quantitative determination of catecholamines is quite helpful for developing nerve physiology, clinical diagnosis of some diseases and controlling medicine in pharmacological research.

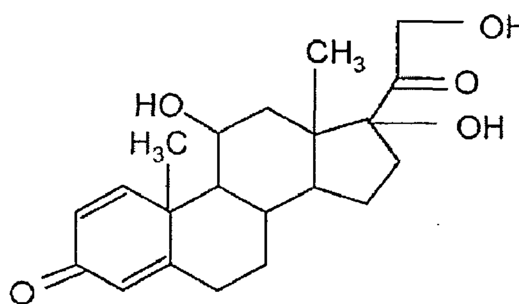
The release of catecholamines in human system also depends on smoking and exercise because these stimulants activates the sympathetic nervous system acting via splanchnic nerves to the adrenal medulla and thus stimulates the release of catecholamines into the blood stream. Therefore, blood plasma and urine samples of smokers and athletes were analyzed to determine catecholamines – epinephrine and norepinephrine concentrations. A broad bump at ~ 250 mV is appeared for the oxidation of epinephrine and norepinephrine at bare EPPGE whereas at MWNT/EPPGE two well-separated peaks at ~150 and ~215 mV are appeared for the oxidation of EP and NE, respectively. The oxidation peak current of both the neurotransmitters increased significantly along with the negative shift of peak potentials using MWNT/EPPGE. The oxidation of both the compounds occurred in a pH dependent, 2e and $2H^+$ process and the electrode reaction followed diffusion controlled pathway. Linear calibration curves were obtained for epinephrine and norepinephrine in the range 0.5 – 100 nM with limit of detections 0.15×10^{-9} and 0.90×10^{-10} M, respectively.

The fourth chapter of the thesis deals with the comparative study of the electrocatalytic activity of two types of CNTs – single walled carbon nanotubes (SWNTs) and multi walled carbon nanotubes (MWNTs) towards electrochemical determinations. A detailed comparison has been made between the response of SWNT and MWNT modified edge plane pyrolytic graphite electrode in terms of several important analytical parameters viz. sensitivity, selectivity and detection limit in order to understand that which one have enhanced electrocatalytic activity. The chapter also discussed the effect of surfactant – cetyltrimethyl ammonium bromide as electrode surface modifier along with single walled carbon nanotubes. The chapter is divided into three sections – first section deals with the voltammetric determination of a new potent calcium antagonist, amlodipine besylate (ADB), in human body fluids mainly focused on the comparison of electrocatalytic activity of MWNTs and SWNTs towards the oxidation of ADB. The second section describes the

simultaneous determination of two important synthetic corticosteroids – prednisolone and prednisone in human body fluids and pharmaceutical preparations at single walled carbon nanotubes modified edge plane pyrolytic graphite electrode (SWNT/EPPGE). The urine and plasma samples of patient undergoing treatment with prednisolone were analyzed so that the method can be used to determine prednisolone and prednisone in doping cases and other clinical purposes. The third section discusses the use of single walled carbon nanotubes-cetyltrimethylammonium bromide nanocomposite film modified edge plane pyrolytic graphite electrode (SWNTs-CTAB/EPPGE) for the determination of betamethasone, a potent pharmaceutical ingredient and doping agent, in urine samples of pregnant women who are undergoing treatment with betamethasone.



Amlodipine besylate



Prednisolone

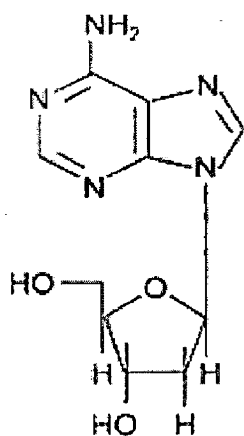
The aim of fifth chapter of the thesis is to resolve the conflicting views and find out the actual reason of electrocatalytic property of fullerene. The effect of embedded metallic impurities (Fe, Cu, Co, Ni) of fullerene, which are accessible to fluids on which fullerene is casted is studied. The fullerene modified glassy carbon electrode was also employed to determine concentration of adenine and 2'-deoxyadenosine (2'-dAdo) in urine samples of carcinoma patient. The chapter is divided into two sections– in the first section the effect of embedded metallic impurities of fullerene; substrate and the application of fullerene-C_{60}-modified electrodes for the determination of nandrolone have been discussed. Second section deals with the simultaneous determination of adenine and 2'-deoxyadenosine in human urine and plasma samples.

Nandrolone, an anabolic androgenic steroid banned in sports by the International Olympic Committee and World Anti-Doping Agency, as it is extensively misused by

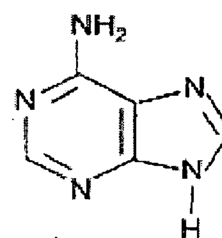
bodybuilders and athletes for the purpose of enhancing athletic performance, has been discussed in the first part of this chapter. Clinically, it is prescribed in the treatment of anaemia, neoplasia including breast cancer, rebuilding of muscles after debilitating disease and treatment of osteoporosis in postmenopausal women. A comparison of edge plane pyrolytic graphite substrate is made with other substrates like indium tin oxide, glassy carbon, gold and basal plane pyrolytic graphite as a substrate for fullerene modification for the determination of nandrolone by square wave voltammetry in phosphate buffer media. Comparative study of voltammetric response of nandrolone at untreated, purified and super-purified fullerene modified edge plane pyrolytic graphite electrode is also carried out to determine the role of embedded metallic impurities of fullerene on determination of nandrolone. It is observed that edge plane pyrolytic graphite electrode serves as best substrate among the studied for casting fullerene. The removal of embedded metals from fullerene shifts the peak potential of nandrolone to more positive potentials and peak current decreases. A linear calibration curve is obtained in the concentration range of 0.01 – 50 nM with a detection limit and sensitivity of 1.5×10^{-11} M and 1.838 $\mu A\ nM^{-1}$, respectively. The developed method was satisfactorily applied to the determination of nandrolone in several commercially available medicinal samples at untreated fullerene – C_{60} – modified edge plane pyrolytic graphite electrode.

Adenine is one of the two purine nucleobases and 2'-deoxyadenosine (2'-dAdo) is one of the purine 2'-deoxyribonucleosides present in deoxyribonucleic acid (DNA) and therefore, both are essential molecules of life and evolution. Adenine is of tremendous biological significance as it is one of the nitrogenous bases found in deoxyribonucleic acid and ribonucleic acid to make up genetic information. It is a component of adenosine triphosphate 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 plays an important role in protein synthesis. 2'-dAdo is a carbohydrate derivative of adenine and the conversion of 2'-dAdo to adenine represent a protective device to control the plasma level of 2'-dAdo when the activity of adenosine deaminase (ADA) is inhibited. In the case of hepatocellular carcinoma the level of adenine has been found to increase considerably. Thus, the simultaneous determination of 2'-dAdo and adenine is the subject of considerable interest especially in human body fluids particularly urine in case of carcinoma. Thus, keeping in consideration the importance of adenine concentration in body fluids studies have been performed to

determine its concentration in urine sample of carcinoma patient using fullerene – C_{60} – modified glassy carbon electrode. Two well-defined anodic peaks at potential of 1248 mV and 994 mV were observed for 2'-dAdo and adenine, respectively. Linear calibration curves were obtained within the concentration range 10 nM – 100 μM for both compounds in 0.1 M phosphate buffer solution having the limit of detection 1.5×10^{-8} M and 4.5×10^{-8} M for 2'-dAdo and adenine, respectively.



2'-deoxyadenosine



Adenine

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Firstly, I have a big sense of gratitude to ***Maa Saraswati*** for giving me the grace, the strength, the wisdom and the means to undertake this Ph.D. programme. This thesis would not have been possible unless God's guiding; enriching and giving me sound mind every day to complete this study. When all hopes, strength and determination to carry on with the research were drained in troubled times, he gave me the insight to realize his strength and presence. Thank you very much Lord ***Guru Jambheshvar Ji*** and I give you all the glory.

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I have frequently made use of the three important websites; Google Scholar, Science-Direct and Sci-Finder for scientific articles with great search controls. I am sincerely thankful to the providers and contributors of these websites. I also wish to thanks anonymous authors whose works formed the basis upon which I built my research ideas. I owe big gratitude to the unsigned reviewers who sent feedback with most interesting suggestions about the original manuscripts.

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LIST OF PUBLICATIONS

[1] R.N. Goyal, S. Bishnoi; "Sensitive voltammetric sensor for the determination of oxidative DNA damage in calf thymus DNA", **Biosensors and Bioelectronics** 26 (2010) 463.

[2] R.N. Goyal, S. Bishnoi; "A novel multi-walled carbon nanotube modified sensor for the selective determination of epinephrine in smokers", **Electrochimica Acta** 56 (2011) 2717.

[3] R.N. Goyal, S. Bishnoi; "Simultaneous determination of epinephrine and norepinephrine in human blood plasma and urine samples using nanotubes modified edge plane pyrolytic graphite electrode", **Talanta** 84 (2011) 78.

[4] R.N. Goyal, S. Bishnoi; "Voltammetric determination of amlodipine besylate in human urine and pharmaceuticals", **Bioelectrochemistry** 79 (2010) 234.

[5] R.N. Goyal, S. Bishnoi; "Simultaneous voltammetric determination of prednisone and prednisolone in human body fluids", **Talanta** 79 (2009) 768.

[6] R.N. Goyal, S. Bishnoi; "Effect of single walled carbon nanotube-cetyltrimethyl ammonium bromide nanocomposite film modified pyrolytic graphite on the determination of betamethasone in human urine", **Colloids and Surfaces B: Biointerfaces** 77 (2010) 200.

[7] R.N. Goyal, S. Chatterjee, S. Bishnoi; "Effect of substrate and embedded metallic impurities of fullerene in the determination of nandrolone", **Analytica Chimica Acta** 643 (2009) 95.

[8] R.N. Goyal, S. Chatterjee, S. Bishnoi; "Voltammetric determination of 2'-deoxyadenosine and adenine in urine of patients with hepatocellular carcinoma using fullerene – C_{60} – modified glassy carbon electrode", **Electroanalysis** 21 (2009) 1369.

[9] R.N. Goyal, S. Chatterjee, S. Bishnoi; "Sensitive voltammetric sensor for determination of flumethasone pivalate, abused for doping by athletes", **Sensors and Actuators B: Chemical** 137 (2009) 676.

[10] R.N. Goyal, A. Tyagi, N. Bachheti, S. Bishnoi; "Voltammetric determination of bisoprolol fumarate in pharmaceutical formulations and urine using single-wall carbon nanotubes modified glassy carbon electrode", **Electochimica Acta** 53 (2008) 2802.

[11] R.N. Goyal, S.P. Singh, S. Chatterjee, S. Bishnoi; "Electrochemical investigations of prednisone using fullerene – C_{60} – modified edge plane pyrolytic graphite electrode", **Indian Journal of Chemistry** 49A (2010) 26.

LIST OF WORKSHOPS / SYMPOSIA / CONFERENCES ATTENDED

[1] **National Workshop on Techniques and Challenges for structure solution in Chemical Crystallography**, August 31 & September 1 (2007), Indian Institute of Technology Roorkee, Roorkee, India.

[2] **International Workshop on Chemical Evolution and Origin of Life**, March 14 – 16 (2008), Indian Institute of Technology Roorkee, Roorkee, India.

[3] **DAE-BRNS International Symposium on Materials Chemistry**, December 2 – 6 (2008), Bhabha Atomic Research Centre, Mumbai, India.

[4] **Golden Jubilee Seminar on Analytical Sciences in Energy and Environment,** November 19 – 20 (2009), Indian Institute of Petroleum Dehradun, Dehradun, India.

[5] **International Analytical Science Congress**, November 24 – 27 (2010), Theme: Analytical Science for Advanced Materials Processing and Environmental Impact Assessment, Cochin University of Science and Technology, Cochin, Kerala, India.

[6] **4TH Conference on Recent Trends in Instrumental Methods of Analysis,** February 18 – 20 (2011), Indian Institute of Technology Roorkee, Roorkee, India.

LIST OF ABBREVIATIONS

CV	Cyclic voltammetry
SWV	Square wave voltammetry
HPLC	High performance liquid chromatography
FE-SEM	Field Emission Scanning Electron Microscopy
ICP-MS	Inductively Coupled Plasma- Mass Spectrometry
CNTs	Carbon nanotubes
SWNT	Single walled carbon nanotube
MWNT	Multi walled carbon nanotube
Ag/AgCl	Silver-silver chloride electrode
GCE	Glassy carbon electrode
PGE	Pyrolytic graphite electrode
EPPGE	Edge plane pyrolytic graphite electrode
BPPGE	Basal plane pyrolytic graphite electrode
ITO	Indium tin oxide
PBS	Phosphate buffer solution
DMF	N, N-dimethylformamide
CTAB	Cetyl trimethyl ammonium bromide
EDTA	Ethylene diamine tetraacetic acid
DNA	Deoxyribose Nucleic Acid
2'-dAdo	2'-deoxyadenosine
EP	Epinephrine
NE	Norepinephrine
SNS	Sympathetic nervous system
CNS	Central nervous system

AA	Ascorbic acid
DP	Dopamine
UA	Uric acid
ADB	Amlodipine besylate
BSP	Betamethasone Sodium Phosphate
E_p	Peak potential
i_p	Peak current
f	Square wave frequency
v	Scan rate
RSD	Relative Standard Deviation
WADA	World Anti-Doping Agency

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Chapter 1

GENERAL INTRODUCTION

Electrochemistry deals with the chemical response of an electrode/electrolyte system to an electrical stimulation. The electrochemical studies can appraise entire behavior of species including concentration, kinetics and reaction mechanisms [1]. Electroanalytical chemistry is an important branch of electrochemistry that utilizes the relationship between chemical phenomena that involves oxidation-reduction and charge-transfer reactions, and the electrical properties that escort these strategies for analytical determinations [2]. Techniques concerned with the interaction between electricity and chemistry that consist of the measurements of electrical quantities such as current, potential, or charge and their relationship to chemical parameters are known as electroanalytical techniques [3]. These techniques have introduced the most promising methods with vast range of applications including industrial, food and water quality control, clinical and biomedical analysis, environmental monitoring alongwith research development [4-8]. Electrochemistry has many advantages that make it an appealing choice for biomedical and pharmaceutical analysis since it has always provided analytical techniques characterized with instrumental simplicity, moderate cost and portability. Additional applications of electrochemistry comprise the determination of electrode reaction mechanisms and redox properties of biomolecules and drugs which can provide insights into their metabolic fate and pharmaceutical activity [9].

Bioelectrochemistry is concerned with the general and basic laws of the electrochemical processes occurring in biological entities, and disregards the particular features of specific systems [10]. For this reason, bioelectrochemical studies are often conducted not on natural objects but on synthetic model systems, viz. artificial membranes. Many of the physical and chemical processes and phenomena that are basic to the vital function of all **biological systems** are electrochemical in nature. Electrochemical and biological both reactions are essentially heterogeneous in nature and specific orientation of the substrate molecules is required for both reactions. Electrochemically, the reaction occurs at the electrode-solution interface and biologically at the enzyme interface. Due to immense resemblance between the electrochemical and biological reactions, it can be implicit that the oxidation/reduction mechanisms taking place at the electrode surface and in the body share similar principles. Hence, the similarities between electrochemical and biological reactions ameliorate the use of electrochemical studies in biological systems. The electrochemical studies provide an extraordinary amount of information about electron transfer reaction of atoms, ions and molecules present in biological system.

In the 1920's, Czech scientist Jaroslav Heyrovsky discovered a form of **voltammetry**, called polarography, in which samples are analyzed by measuring current as a function of the applied electrical potential. A quarter century later, on 26 October 1959, Jaroslav Heyrovsky was awarded the Nobel Prize in Chemistry for his discovery of the polarographic methods of analysis [11]. The voltammetric methods used today in laboratories were made possible by recent advancement in instrumentation, computerized processing of analytical data, and in particular by innovative electrochemists. Voltammetry is an electrochemical technique in which the current-potential behaviour at an electrode surface is measured. The potential is varied in some systematic manner to cause electroactive chemical species to be reduced or oxidized at the electrode. This current is proportional to the concentration of the electroactive component present in the test solution. The current measured is usually the current due to a specific oxidation or reduction process involving the analyte species at the surface of one of the electrodes. The current corresponding to the quantity of material transported by diffusion and reacting at the electrode surface is measured. A three-electrode system includes a working electrode at which the oxidation or reduction process of interest occurs, a reference electrode – saturated calomel electrode (SCE) or silver-silver chloride electrode (Ag-AgCl) and an auxiliary or counter electrode which carries the bulk of the current. In the last several decades voltammetric methods have become a popular tool for the study of electrochemical reactions [12], electrochemically generated free radicals [13], enzymatic catalysis [14], solar energy conversion [15], environmental monitoring [16], industrial quality control [17] and for the determination of trace concentrations of biological and clinically important compounds [18, 19].

The indigence of highly sensitive and selective detecting agents having practical applications and momentous advantages over conventional technologies attracted researchers towards **nanomaterials** based technologies. Last few years have seen a worldwide outburst of research interest of electrochemists at the interface of nanotechnology and bioelectrochemistry. The implementation of nanoscience and nanotechnology achievements in bio-electroanalysis ameliorates the current electroanalytical techniques and methodologies by providing novel sensing system which enhances the performance of bioanalytical assay. The technological advancement in electrochemistry makes it possible to study and understand the principles of nanoscience and nanotechnology. The present day research interest towards nanotechnology is driven by

the desirable properties offered by nanomaterials. As the size of any particles reduced, surface/volume ratio of the particle increases considerably and then surface phenomenon predominates over the chemistry and physics of the bulk material. When the size of the materials reach the nanometer regime and approaches the size of biomolecules then they directly interact with individual biomolecule in contrast to conventional macro- and micro-devices which deal with assembly of relatively large amount of samples. Such properties are more sensitive to the environment and target molecules in the samples. Further, the major factor which attracts application of nanomaterials in sensor research is their tailorable morphology which offers excellent prospects for designing novel **sensing systems** and improves the recitation of the bioanalytical assay [20]. The interdisciplinary trend of present day sensor research makes it clear that the development in this direction may revolutionize the field of clinical, biomedical, pharmaceutical, environmental and industrial research.

The development of chemical sensors using nanomaterials is currently one of the most active areas of analytical research. Chemical sensors consist of a transduction element covered with a chemical or biological recognition layer. This layer interacts with the target analyte and the chemical changes resulting from this interaction are translated by the transduction element into electrical signals. Ideally, such a device is capable of responding continuously and reversibly without perturbing the sample. By combining the sample handling and measurement steps these sensors eliminate the need for sample collection and preparation. Electrochemical sensors are powerful tools in analytical chemistry which represent an important subclass of chemical sensors in which an electrode is used as the transduction element. Such devices have a leading position among sensors presently available, reached the commercial stage, and have found a vast range of important applications in the fields of clinical, industrial, environmental, and agricultural analyses [21-23]. Research pertaining to electrochemical sensors is proceeding in a number of directions as electrochemical biosensors, affinity biosensors and gas sensors.

Voltammetric sensors examine the concentration effect of the detecting species on the current-potential characteristics of the reduction or oxidation reaction involved. The mass transfer rate of the detecting species in the reaction onto the electrode surface and the kinetics of the faradic or charge transfer reaction at the electrode surface directly affect the current potential characteristics. The electrode reaction kinetics and the mass transfer processes contribute to the rate of the faradic process in an electrochemical cell. This

provides the basis for the operation of the voltammetric sensor. The construction of the voltammetric sensors is very robust that makes it suitable in different areas of industrial applications. The voltammetric electronic tongue (taste sensor) has very promising features as a general tool for wet-end control, hence, been used successfully for the evaluation of pulp samples [24]. It has also been used to follow the rinsing process in washing machines, dishing and cleaning processes [25]. Other important industrial applications of voltammetric tongues consist of their use as a monitoring device in drinking water production plants, dairy industry and for the detection of microbial activity [26-29]. A company SensET AB has commercialized the voltammetric electronic tongue [30] in 2001 and now offers a continuous measurement system for chemical oxygen demand (COD). Although, voltammetric techniques are well established but they still have many development possibilities. Interesting areas include miniaturization, use of microelectrodes and development of continuous measurement system for heavy metal (e.g. Cd, Hg, and Pb) detection in soil samples to check microbial activity and water quality.

In voltammetric studies addition of surface-active agents greatly enhances the peak response by increasing sensitivity and decreasing detection limit [31-33]. The combination of the unique properties of carbon based nanomaterials viz. fullerenes and carbon nanotubes (CNTs) having the powerful recognition properties of biomolecules and the known advantages of electrochemical techniques represents a very good alternative for the development of (bio) sensors able to address future biosensing challenges in clinical diagnosis, environmental monitoring, security and doping control [34-36]. Fullerenes and carbon nanotubes with well-defined nanoscale dimension and unique molecular structure can be used as bridges linking biomolecules to macro/micro- solid state devices so that the information of bioevents can be transduced into measurable signals [37]. Exciting new sensing concepts and devices with extremely high sensitivities have been demonstrated using CNTs [38]. Owing to well-defined structure, chemical stability and the electrocatalytic activity toward many substances CNTs are extensively used as the carrier platforms for constructing various electrochemical sensors. CNTs modified electrodes offer excellent electroanalytical properties such as wide potential windows, low background current and good biocompatibility [39]. The origin of electro catalytic properties of nanotubes has been assigned to the embedded metal impurities in CNT samples and edge-plane-like defects which are present at the open ends of nanotubes [40, 41]. A large surface

area of nanotubes modified electrodes affords efficient adsorption of many substances and a high length to radius ratio (ca. 1000:1) of nanotubes also controls their properties in desired direction.

Biomolecules have significant consequences in transmission of genetic information in addition to their several important metabolic functions. Intracellular levels of biomolecules play an important role in human physiology because their altered concentrations cause various metabolic disorders in human system. Determination of biological compounds of physiological importance always remains a challenging task for researchers as their concentration in biological fluids is normally very low. However, in case of metabolic disorders and diseases, the concentration of these biomolecules gets abnormally altered. In such cases, detection of their concentration level proves out to be of diagnostic value. The ability to measure extremely small amounts of specific biomarkers at molecular levels is highly desirable in biomedical research and healthcare. Quantification of common drugs is important for pharmaceutical industry, and studies on doping agents is needed to keep a check on the doping cases in competitive games. Current technologies rely on well-equipped central laboratories for molecular diagnosis which are expensive and time consuming and often cause delay in medical treatments. Hence, still there is an expanding demand of design small, fast, less expensive and simple sensors for molecular analysis. Electrochemical quantification of variety of biomolecules, medicines and doping agents using electrodes modified with nanocarbon/nanoparticles has lead to worldwide explosion of research interest due to promising advantages that they offer. Nanomaterials modified sensors have been found to show consistently good results with high sensitivity and selectivity. Hence, an endeavor has been made in the present investigation to develop simple, selective and sensitive electroanalytical methods for qualitative and quantitative analysis of biologically important compounds and drugs using nanomaterial modified electrodes. The foremost aim of the present investigation is to assess the utility of voltammetric methods using various electrodes and surface-active agents for the determination of biomolecules and pharmaceuticals with low running cost, high speed and sensitivity.

1.1 SUCCINCT OVERVIEW OF THE METHODOLOGY USED IN THE PRESENT INVESTIGATIONS

The use of electroanalytical methods has found a gigantic range of applications in environment monitoring, industrial quality control, clinical and biomedical analysis. A brief description about the fundamentals of the various electroanalytical techniques used in the present investigation is presented in upcoming paragraphs.

1.1.1 Cyclic voltammetry

Cyclic voltammetry (CV) is the most widely used electroanalytical technique offering a wealth of information for the study of redox couples, reaction kinetics and the mechanism of many electrochemical reactions [42, 43]. 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 outcome 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. In particular, it offers a rapid location of redox potentials of the electroactive species and convenient evaluation of the effect of media on the redox process.

Cyclic voltammetry consists of scanning linearly the potential of a stationary working electrode in an unstirred solution using a triangular potential waveform as depicted in **Fig. 1.1**. 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.

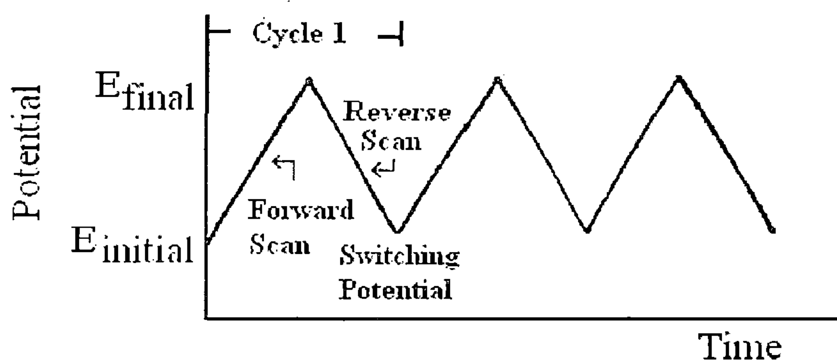


Fig. 1.1 Potential-time excitation signals in cyclic voltammetric experiment.

The response of a reversible redox couple during a single potential cycle is illustrated in **Fig. 1.2**. It is assumed that only the oxidized form O is present initially. Thus, a negative-going potential scan is chosen for the first half-cycle, starting from a value where no reduction occurs. As the applied potential approaches the characteristic E^0 for the redox process, a cathodic current begins to increase, until a peak is reached. After traversing the potential region in which the reduction process takes place the direction of the potential sweep is reversed. During the reverse scan, R molecules generated in the forward half cycle, and accumulated near the surface are reoxidized back to O and an anodic peak results. The characteristic peaks in the cyclic voltammogram are caused by the formation of the diffusion layer near the electrode surface.

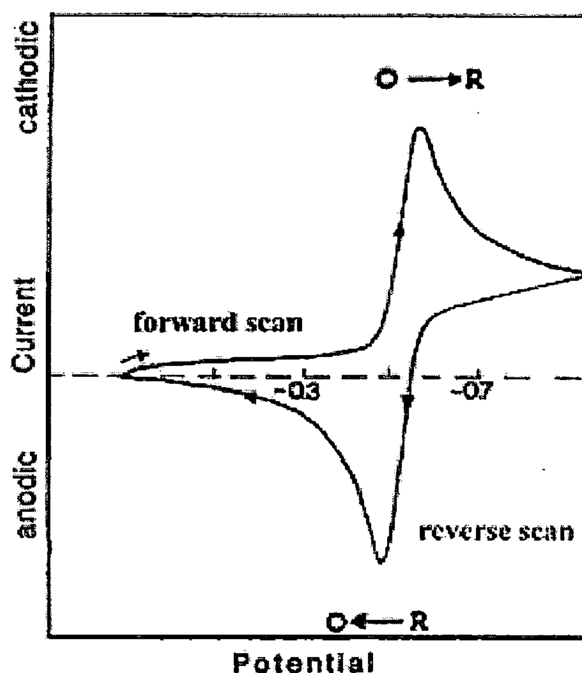


Fig. 1.2 Typical cyclic voltammogram for a reversible O + ne$^-$ ⇌ R redox process.

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

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

where i_p is peak current in μA, n is the number of electrons, A is the electrode area (cm^2), C is the concentration (mol/cm^3), D is the diffusion coefficient (cm^2/s) and v is the scan

rate (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.

However, the use of cyclic voltammetry has been restricted primarily to the gathering of qualitative or diagnostic purpose. Quantitative determinations are best obtained by using pulse techniques viz. square-wave voltammetry [44, 45]. By comparison these latter techniques offer increased sensitivity over cyclic voltammetry due to their ability to effectively separate charging current, which contributes largely to the background, from the faradic current. The interpretation of the peak readout is also much simpler in pulse techniques. In multi-component analysis well-defined peaks are simpler to resolve as opposed to the semi-peak readout for cyclic voltammetry. Thus, it is more advantageous to exploit pulse techniques coupled with cyclic voltammetry to obtain inclusive information about redox system.

1.1.2 Square-wave voltammetry

Square wave voltammetry (SWV) 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 as shown in **Fig. 1.3** [46]. 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 sigmoid curve typically found for normal pulse voltammetry. Excellent sensitivity accrues from the fact that the net current is larger than either the forward or reverse components, since, it is the difference between them. The sensitivity is higher than that of other pulse techniques in which the reverse current is not used. Coupled with the effective discrimination against the charging background current very low detection limit (near 1×10^{-8} M) can be attained.

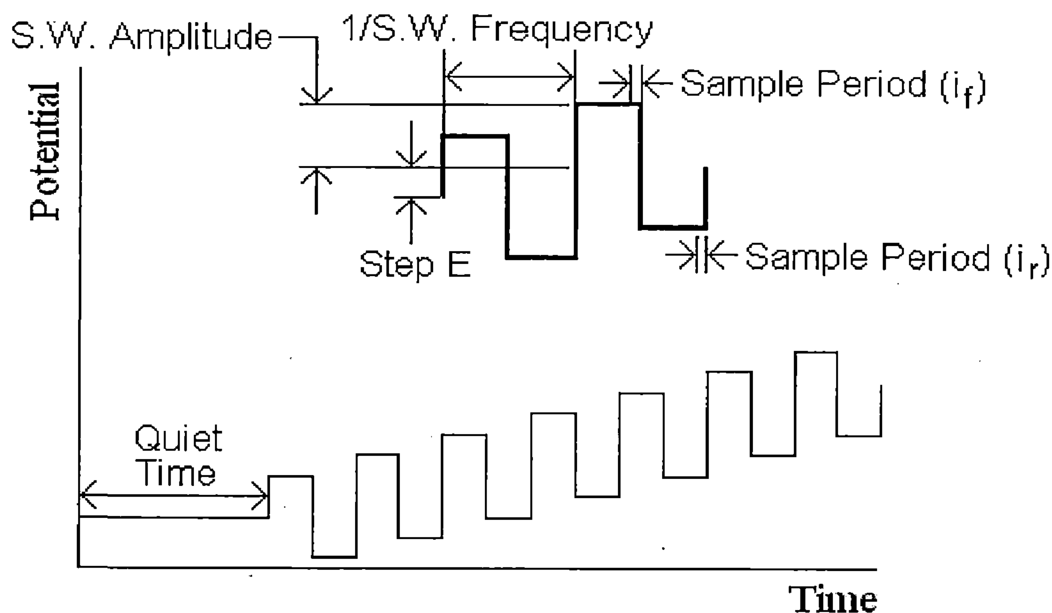


Fig. 1.3 Potential wave form for square wave voltammetry.

Another important advantage of square wave voltammetry is its speed. Frequencies of 1 to 100 square-wave cycles per second permit the use of extremely fast potential scan rates. As a result the analysis time is drastically reduced and the entire voltammogram can be recorded within a few seconds. Thus, SWV offers the advantages of high sensitivity, great speed and low detection limit alongwith symmetric peak. In the present dissertation, the electrochemical analysis of various biomolecules and drugs was carried out by different voltammetric techniques using bioanalytical system (BAS CV-50 W) shown in **Fig. 1.4**.

Fig. 1.4 Bioanalytical system used for the electrochemical studies.

1.2 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

High Performance Liquid Chromatography (HPLC) was developed in the late 1960's. Today it is widely applied for the separation and purification in a variety of areas including pharmaceuticals, biotechnology, environmental, polymer and food industries [47-49]. The structural analysis of biomolecules [50, 51], analysis of human blood proteins [52] and biochemical analysis [53] are also some important fields of its applications. HPLC is accomplished by injection of a small amount of liquid sample into a moving stream of liquid (mobile phase) that passes through a column packed with particles of stationary phase. Separation of a mixture into its components depends on different degrees of retention of each component in the column. The extent to which a component is retained in the column is determined by its partitioning between the liquid mobile phase and the stationary phase. In HPLC this partitioning is affected by the relative solute/stationary phase and solute/mobile phase interactions. Thus, changes in mobile phase composition can have an

enormous impact on separation. The compounds of different mobilities exit from the column at different times and thus have different retention times (Rt) in the form of peaks. Thus, the retention time is the time difference between injection and detection. The detector, considered as the "soul" of a HPLC system, is a device that senses the presence of components different from the liquid mobile phase and converts that information to an electrical signal. Therefore, selective detectors are required to minimize interference from unwanted components. The Ultraviolet-Visible (UV-Vis) detector is more selective, sensitive and being able to detect amounts as low as 10^{-10} g/mL. In the present investigation HPLC having UV detector was used to validate the data obtained from voltammetric methods in order to prove the accuracy of results obtained.

1.3 CONVENTIONAL ELECTRODES

The term working electrode is used for the electrode at which the reaction of interest takes place. Since, the performance of the voltammetric method is strongly influenced by the material of working electrode, hence, it is very important to choose appropriate geometry and composition of electrode material for a particular study. As the working electrode material influences the chemical steps and the electron transfer process involved in the detection of the analyte [54, 55], the physical form of the electrode influences the diffusion process. The selected working electrode should provide high signal-to-noise characteristics and reproducible responses. Thus, its selection depends on the redox behavior of the investigated compound and the background current over the potential region required for the measurement. In addition, the potential range, surface reproducibility, mechanical properties, cost, availability, electrical conductivity, and toxicity are some other important criteria for choosing the working electrode material. Further, the requirements imposed on electrodes also include electrochemical inertness over a broad interval of potentials, high overvoltage of hydrogen and oxygen evolution, low residual current and the possibility of simple regeneration of the surface. Despite the difficulty of controlling their surfaces in a reproducible manner and low overvoltage of hydrogen, the increasing popularity of solid electrodes over mercury electrode can be attributed to the facts that the oxidation of many organic molecules cannot be studied by the mercury electrode because of its limited anodic potential range, toxicity and strong adsorption with some analytes which creates unsatisfactory waves. A great variety of solid electrodes have been employed in different voltammetric techniques over the years.

Metal based electrodes such as gold, platinum and silver are currently in widespread use in electroanalytical chemistry because of their high conductivity, broad potential range and suitability for various sensing and detection applications. Silver was the first noble metal solid electrode used for voltammetric analysis [56]. Platinum is used most frequently as the electrode material since the main advantages of the use of platinum in electrochemistry comprises clear separation of the potential regions for hydrogen and oxygen adsorption and excellent corrosion resistance coupled with high catalytic activity [57]. Gold electrodes are also widely used for the determination of some trace elements [58, 59]. The main drawbacks of noble metal electrodes are that they form an oxide layers or dissolution of metals and exhibit low hydrogen overvoltage, which strictly limit their uses.

Carbon based electrodes encompass slow electron transfer rate resulted in slow kinetics than metal electrodes leading to a wide potential range especially in the anodic direction [60-62]. Other important benefits of these electrodes include chemical inertness, low background current, relatively high oxygen and hydrogen overvoltage, rich surface chemistry and low cost. Carbon based materials do not interact with analytes or deposited compounds during electrolysis that rules out the appearance of a systematic error caused by such interactions. These electrodes have surface functional groups for chemical modification and controllable surface activity resulting from pretreatment [63, 64]. Carbon paste electrodes are an example of the more general class of composite electrodes in which chemically useful functionalities can be introduced during physical mixing of graphite and pasting liquids. The important drugs and biomolecules including diazepam oxazepam, chloroquine, pefloxacin, lansoprazole omeprazole, quercetin, riboflavin, tryptophan, ethinylestradiaol, N-acetylcysteine, ascorbic acid and dopamine have been determined recently in urine, plasma, serum and different dosages forms using carbon paste electrode [65-74]. Diamond is also promising candidate for electrode material to be used in electroanalytical chemistry especially in pharmaceutical analysis because of its extraordinary chemical stability even under extreme conditions such as strongly acidic media [75-77]. One other form of carbon based electrodes is boron doped diamond electrode that consists of diamond in which approximately one atom in a thousand has been replaced by boron giving a material with metallic conductivity. Diamond is one of the nature's best insulators but when doped with boron the material can possess either semi-conducting or semi-metallic electronic properties depending on the doping level. Boron is

easily incorporated, heavily doped and produce less resistant films and consequently these electrodes have colossal applications in electrochemical studies [60, 77, 78].

Carbon fiber electrodes are extensively applied in the electrochemical determination of a wide variety of species due to various advantages that they offer [79-81]. An important form of carbon based electrode is glassy carbon electrode (GCE) that has been employed for the analysis of human urine, serum, gastric fluids, breast milk and dosage forms containing various drugs such as amlodipine [82], valacyclovir [83], cefixime [84], atenolol [85] and prednisolone [86]. GCE has also been used in the detection of insulin [87], simultaneous detection of natural antioxidants [88] and sonoelectrocatalysis of oxygen reduction and hydrogen peroxide formation [89]. Pyrolytic graphite electrode (PGE) is the most versatile electrode in electrochemical investigations due to its wide available potential window [90]. PGE has been successfully utilized in the direct electron transfer of haemoglobin and myglobin [95], oxidation of deoxyribonucleic acid [96], electrochemical investigation of human adrenodoxin [97], detection of epinephrine [98], NADH detection [99], chlorine reduction [100] and nitrogen dioxide detection [101]. The edge plane pyrolytic graphite electrode (EPPGE) exhibits elegant results with adequate sensitivity without any additional procedure for renewal of the electrode surface except rubbing it on an emery paper. Our laboratory is also actively studying on the electrochemical determination of variety of biomolecules and drugs using EPPGE [91-94]. In the present investigation pyrolytic graphite electrode and glassy carbon electrode have been utilized actively as the working electrodes hence, general characteristics of both the electrodes are summarized below.

A polycrystalline form of carbon having high degree of orientation is known as pyrolytic graphite. It is formed by the thermal decomposition (pyrolysis) of carbonaceous gases in the temperature range 1900 – 2500 °C under reduced pressure. It represents hard, poreless, chemically stable and clearly pronounced anisotropic properties. The structure of graphite presents lamellic planes of sp^2 carbon organized in hexagons, with a very high degree of delocalization of π electrons giving a good electrical conductivity, held together by weak Van der Waals forces. Graphite powder is mixed with suitable filler and then either physically or chemically bonded to form a conductive solid composite. Pretreatment and polishing procedure play important roles in increasing or decreasing the electron transfer rates depending on exposure of edge and basal planes. Both edge plane and basal plane pyrolytic graphite electrodes are constructed from highly ordered pyrolytic graphite

(HOPG). The basal plane surface of an HOPG electrode consists of layers of graphite which lie parallel to the disc surface and with an interlayer spacing of 3.35 Å. Surface defects occur in the form of steps exposing the edges of the graphite layers (**Fig. 1.5**). Conversely, a basal plane pyrolytic graphite electrode (BPPGE) is fabricated such that the layers of graphite lie parallel to the disc surface [102]. Both, edge and basal plane pyrolytic graphite share the basic structure of a six-member aromatic ring and sp^2 bonding but differ in the relative density of the edge and basal plane toward electron transfer and adsorption. The two planes, edge and basal, exhibit completely different electrochemical properties due to the nature of chemical bonding in graphite. The electrode consisting entirely of edge plane viz. edge plane pyrolytic graphite electrode shows faster electrode kinetics, high sensitivity and low detection limit as compared to an electrode consisting mainly of basal plane [103].

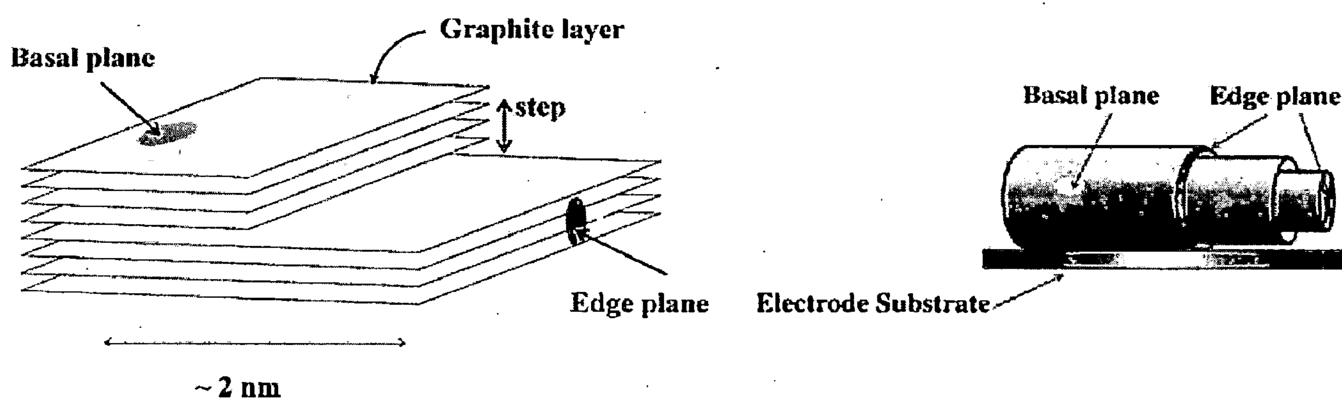


Fig. 1.5 Representation of a crystal of highly ordered pyrolytic graphite surface.

Glassy carbon is comprised of a structure of interwoven thin ribbons of cross linked graphite like sheets (**Fig. 1.6**). It is prepared by means of a carefully controlled heating program of a premodeled polymeric (phenol-formaldehyde) resin body in an inert atmosphere [104]. The carbonization process is carried out very slowly over the 300 – 1200 °C temperature range to insure the elimination of oxygen, nitrogen, and hydrogen. Because of its high density and small pore size no impregnating procedure is required. Then surface pretreatment is employed to create active and reproducible glassy carbon surface and to enhance the analytical performance [105]. Such treatment is usually achieved by polishing (to shiny "mirror-like" appearance) with successfully smaller alumina particles (0.05 μm) on smooth polishing cloth. The electrode should then be rinsed with deionized

water before use. The improved electron-transfer reactivity has been attributed to the removal of surface contaminants, exposure of fresh carbon edges and an increase in the density of surface oxygen groups. Unlike many non-graphitizing carbons it is impermeable to gases and also highly resistant to acid attack. Glassy carbon possesses isotropic properties and does not require a particular orientation in the electrode device as in the case of pyrolytic graphite.

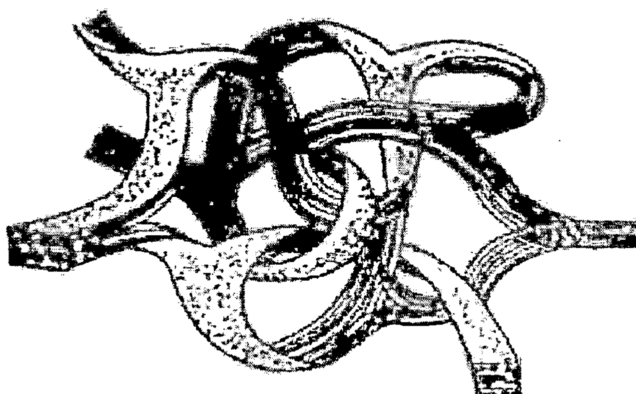


Fig. 1.6 The structure of glassy carbon with interwoven thin ribbons of cross linked sheets.

1.4 MODIFIED ELECTRODES

Modified electrodes represent a modern approach to electrode systems which rely on the placement of a reagent onto the electrode surface to impart the behavior of that reagent to the modified surface. Such deliberate alteration of electrode surface can meet the needs of many electroanalytical problems and forms the basis for new analytical applications and different sensing devices. Recently, the field of modified electrodes has become incredibly popular with large number of applications in industry, quality control of drugs and food, determination of pharmaceutical dosage forms and environmental monitoring. Desirable characteristics for modified electrodes in voltammetric experiments comprise the diminution in the overpotential of the analyte under investigation, increased stability of the electrode response coupled with increments in peak heights facilitating lower detection limits and increased specificity compared to the bare electrode. Modified electrodes can benefit analytical applications by various ways which incorporate acceleration of electron

transfer reactions, preferential accumulations or selective membrane permeation. Such steps can impart higher selectivity, sensitivity and stability to electrochemical devices and these analytical applications and improvements have been extensively reviewed [106-111]. Many other important applications including controlled release of drugs, electro-synthesis and corrosion protection are also benefited from the rational design of electrode surfaces.

Electrode modification with catalytically active molecules has become a very active field of research over the past two decades. Gold nanoparticles exhibit attractive properties as electrode surface modifiers by improving the electrode conductivity and enhancing the analytical sensitivity and selectivity [112, 113]. Wang *et al.* [114] reported a new approach to construct a nano-Au self-assembly gold electrode which was used successfully for the determination of epinephrine. Hu *et al.* [115] also reported the fabrication of a modified electrode based on the self-assembly of gold nanoparticles on cysteamine film which has been bound to the surface of a glassy carbon electrode. The modified electrode exhibited an excellent reproducibility, sensibility and stability for determination of dopamine in the presence of high concentrations of ascorbic acid. Goyal and co-workers have successfully studied the electrochemical behavior of various pharmaceuticals [116-119] using a gold nanoparticle-modified indium tin oxide (nano Au/ITO) electrode. Karam and Halaoui reported the fabrication of Pt nanoparticles arrays assembled in low densities in polyelectrolyte for the detection of hydrogen peroxide (H_2O_2). This finding is especially useful for applications where continuous H_2O_2 sensing is required as it simplifies construction, reduces the amount of Pt needed and thus lowers the cost of sensor fabrication [120]. Wen and colleagues synthesized amorphous iron/nickel/platinum nanoparticles and applied them to a glassy carbon electrode for the detection of thiols [121]. The size of the nanoparticles was tuned by varying heating temperature during phase transfer synthesis. The resulting sensors exhibited enhanced electrocatalytic activity toward cysteine oxidation due to both the alloy material and nanoparticle size with a limit of detection for cysteine as 50 nM.

Over the past two decades electroanalytical techniques based on polymer modified electrodes have attracted broad interest of scientists engaged in pharmaceutical analysis [122-124]. The voltammetric behavior of pyrantel pamoate was studied in the Britton-Robinson buffer system at a composite polymer membrane working electrode by cyclic voltammetry for the determination of drug in pharmaceutical formulation. A well-defined cathodic peak was observed for the pyrantel pamoate in the entire pH range. The results

indicate that the current increases steadily with concentration and scan rate, and the electrode reaction followed diffusion controlled pathway [125]. The modification of electrode surface by Nafion film [126] enhanced the analytical signal intensity and simultaneously protected the surface of the working electrode alongwith conferring greater stability and higher reproducibility towards the electrochemical determinations. Zheng and co-workers reported the sensitive and simultaneous determination of dopamine, ascorbic acid and uric acid at ordered mesoporous carbon (OMC)/Nafion composite film [127]. Jain *et al.* also reported a composite polymer surface coated on a tin oxide which offers dramatic improvement in the stability of voltammetric measurement of pyridostigmine bromide compared to individual tin oxide [128]. Ciobanu and co-workers reported the fabrication of ultramicroelectrode (UME) biosensors for glucose and lactate based on glucose oxidase and lactate oxidase, respectively [129]. The glucose UME biosensor was used successfully to record profiles of glucose uptake. Likewise, the lactate UME biosensor was employed to record the lactate production. Recently Nasirizadeh *et al.* had fabricated a highly efficient noradrenalin biosensor on the basis of hematoxylin electrodeposited on a glassy carbon electrode. The differential pulse voltammetric peaks for noradrenaline and acetaminophen oxidation at the hematoxylin biosensor surface are clearly separated from each other when they coexisted in the physiological pH (pH 7.0) [130]. Batista and co-workers utilized iron tetrapyridinoporphyrazine as a catalyst for the detection of estradiol valerate (EV) at a carbon paste electrode [131]. The electrode exhibited a dynamic linear range of 45-450 μM and a detection limit of 13 μM. The authors successfully applied the electrode for detection of EV in commercial preparations.

The effect of surfactants like sodium dodecyl sulfate (SDS), cetyltrimethylammonium bromide (CTAB) and Triton X-100 (TX-100) was studied by mobilizing and immobilizing methods for the determination of sodium levothyroxine. The concentration effect of all the three surfactants was studied and it was observed that SDS shows excellent enhancement in both oxidation peak and reduction peak currents [132]. Gholivand and Amiri fabricated a nuclear fast red (NFR)/porous polypyrrole sensor capable of detecting methyldopa (MDA) and ascorbic acid [133]. A polypyrrole film was electrosynthesized in the presence of NFR on a gold electrode and overoxidized to increase porosity of the sensor. The anionic NFR dopant increased selectivity over interferences such as oxalate, urea, and citrate. The resulting sensors exhibited limits of detection for MDA and ascorbic acid as 50 nM and 5.8 μM, respectively. A carbon-paste electrode spiked with

ferrocenedicarboxylic acid (FDCMCPE) was constructed by incorporation of ferrocenedicarboxylic acid in a graphite powder–paraffin oil matrix. It has been shown by direct current cyclic voltammetry and double-step chronoamperometry that this electrode can catalyze the oxidation of ampicillin (AMPC) in aqueous buffered solution. It has been found that under optimum conditions (pH 10.0) the oxidation of AMPC occurred at a potential of about 480 mV in cyclic voltammetry on the surface of modified carbon paste electrode. This method was also explored as a selective, simple and precise electrochemical sensor for the determination of AMPC in real samples such as drugs and urine [134]. Mwilu *et al.* reported the identification and quantification of *Bacillus globigii*, a spore forming nonpathogenic stimulant of *Bacillus anthracis*, using a label-free metal-enhanced electrochemical immunosensor [135]. Several synthetic zeolites such as mazzite, mordenite, zeolite L, zeolite beta and MCM-41 were tested as electrode surface modifiers in voltammetric determination of tryptophan. It was found that the addition of zeolite beta to the carbon paste electrode would generate the peak current of tryptophan. The interference of several species especially amino acids was tested. The proposed method was applied successfully to the determination of tryptophan in pharmaceutical formulations [136].

As a new type of carbonaceous materials, carbon nanotubes (CNTs) possess some unique properties that are much different from the conventional scaled materials. Such properties include well-defined tubular structure of nanosizes, functional surfaces, modifiable ends and sidewalls, excellent chemical stability, strong electrocatalytic activity and excellent biocompatibility [137]. The three dimensional special architecture of the CNTs can lead to a high loading of electrocatalysis or biomaterial onto the solid substrate and thus can enhance the efficiency for (bio) electrocatalysis. As tubular nanomaterials, the key advantages of CNTs are their small diameter and huge length to diameter ratio that allows them to be used as molecular wires for facilitating electron transfer between biomolecules and electrodes with ultra-sensitivity. These special properties foresee their promising applications in electroanalytical chemistry make CNTs ideal candidates for constructing sensors with high performances. Peng *et al.* suggested that the intrinsic electronic properties of carbon nanotubes remain unaffected even when they are in direct contact with water by an *ab initio* study of water adsorbed on single walled carbon nanotubes shows purely repulsive interaction without any charge transfer [138]. This study revealed new avenues for application of carbon nanotubes modified sensors in aqueous medium. The modification of electrodes with CNTs has been observed to apparently

improve the responses of substrates from small H_2O_2 molecules to huge redox proteins. The electron transfer and the direct electrochemistry of redox proteins at CNTs based electrochemical sensors is well reported [139, 140]. Excellent improvements in the electrochemical behavior of biologically important compounds such as dopamine and ascorbic acid [141, 142], quercetin and rutine [143], tryptophan [144], tyrosine [145], procaine [146] and metformine [147] have been demonstrated at CNTs modified electrodes. CNTs modified electrodes were also utilized to determine hemoglobin in bovine blood [148]. Multi walled carbon nanotubes modified carbon paste electrode (MWNT/CPE) was used to study the electrochemical behavior of bergenin [149]. The modified electrode showed excellent electrocatalytic activity in lowering the anodic overpotential and remarkable enhancement in anodic peak current of bergenin as compared to the electrochemical performance obtained at CPE. A voltammetric method was developed for the determination of tetracycline (TC) by using an ionic liquid (IL, 1-octyl-3-methylimidazolium- hexafluorophosphate) – multi walled carbon nanotubes film coated glassy carbon electrode (GCE). The results indicated that both IL and MWNT could facilitate the TC oxidation and the electrode had good reproducibility. It was successfully applied to the detection of TC in egg and pharmaceutical samples [150]. The single walled carbon nanotube (SWNT) modified gold detector for microchip CE has been constructed and successfully used for the detection of *p*-aminophenol, *o*-aminophenol, dopamine and catechol [151]. SWNT modified glucose biosensors exhibited a wider dynamic range and greater sensitivity in glucose determination [152]. A nanocomposite of poly- Nile blue with SWNTs demonstrated the ability to electrocatalyze the oxidation of NADPH at a very low potential (– 80 mV versus SCE) with a substantial decrease in the overpotential by more than 700 mV as compared with the bare GCE [153].

Fullerene science is one of the fastest growing areas of research in chemistry, physics, and material science. One imperative field of their application is their use as mediators in electrochemistry for the chemical modification of electrodes in electrocatalysis. Fullerene C_{60}/C_{70} modified electrodes catalyze the redox reaction of variety of compounds due to the formation of more conducting C_{60}^{n-} species during partial reduction of C_{60}, which help electron transfer at the interface [154, 155]. Fullerene anions (reduced fullerene) abstract proton from biomolecules and have some special properties like nano size and higher surface area to volume ratio. A very interesting feature of the partial reduction of fullerene films in aqueous solutions is the proposed 'sandwich like' reduction.

Partially reduced fullerene films have a structure with a polar inner and outer surface, while the inside is non polar. The structure very much resembles to a biological membrane, thus raising the possibility of using fullerene as solid state modifiers to study the electrochemistry of biomolecules [156]. Therefore, fullerene modified electrodes meet the conditions essentially required for the analysis of biomolecules which consist of excellent reproducibility, high sensitivity, wide potential range and high stability in biological samples [157]. The performance of fullerene – C_{60} – modified electrodes has been reported to produce electrocatalytic responses compared to the underlying electrode for certain target analytes. Jehoulet *et al.* demonstrated the formation of C_{60} film casted on an electrode surface by evaporation of fullerene solutions unveiled the possibility to study the detailed aspects of its electrochemistry [158]. Szucs and coworkers explored gold surfaces for the electrochemical determination of cytochrome c [159]. Further, the electrochemical response of cytochrome *c* was determined by Csiszar *et al.* using fullerene C_{60} modified electrodes with optimum results [160]. Tan, Bond and co-workers reported the electrochemical oxidation of L-cysteine in aqueous solution using C_{60} – modified glassy carbon electrode [161]. Goyal *et al.* reported the electrochemical oxidation of uric acid to be mediated by C_{60} supported on glassy carbon electrodes. The overlapping voltammetric response of uric acid and ascorbic acid observed at the bare glassy carbon electrode being resolved into two well-defined voltammetric peaks with a potential difference of ~150 mV facilitated through the introduction of C_{60} on the glassy carbon electrode surface [162]. Also, the same group further studied the effect of surface modification by C_{60} for the voltammetric determination of various biomolecules and drugs and reported that the use of C_{60} modified electrodes decreases the peak potential and increases the peak current alongwith improving detection limit and sensitivity [163-166].

1.5 ORIGIN OF ELECTROCATALYTIC ACTIVITY OF CARBON NANOTUBES AND FULLERENES

1.5.1 Carbon nanotubes

There has been an explosion of modifying electrodes with carbon nanotubes in the search for the origin of electrocatalytic responses of nanotubes [167-169]. It is well documented that such improvements are due to the unique structure of the carbon nanotubes where the presence of edge plane like-sites/defects are the origin of heterogeneous charge

transfer [103, 170]. In this sense, Compton's group reported a very interesting work dealing with the investigation about the reason why CNTs present the enhanced electrocatalytic activity and they proposed that this activity is due to the presence of edge-plane like sites located at the end and the "defects" areas of the tubes [103, 167]. Further, Ab-initio calculations demonstrated that the improvement in the electron transfer is due to the curvature of the tubes that originate changes in the energy bands close to the Fermi level [171]. The presence of pentagonal defects produce regions with charge density higher than those observed in the region of hexagonal graphite, either in planar or in tubular structures demonstrating the connection between topological defects and CNTs electroactivity [171]. Carbon nanotubes can be seen as the graphene sheets rolled into tubes. The cap regions of nanotubes may be more reactive due to the much higher curve strain than the sidewall. Based on their specific structures, two distinct surface regions exist in carbon nanotubes (CNTs): the sidewalls and the ends. The opening of the ends by physical/chemical treatments on carbon nanotubes produces a variety of oxygen-containing groups [172]. Moreover, the intact CNT sidewalls resemble the basal planes of pyrolytic graphite and can be regarded electrochemically inert to electroactive species. The apparent improved electron transfer at the intact nanotube sidewalls is attributed to the higher stains than the basal planes. As for the opened caps, the presence of defects and oxygen-containing functional groups makes them possess similar electrochemical properties to those of edge planes of pyrolytic graphite. Thus, the introduction of edge-like defect sites and oxygen-containing functional groups at both the caps and the sidewalls by chemical or physical treatments can significantly improve the electrochemical properties of CNTs by changing the electronic structures, surface states and the wet ability of the sidewalls [173]. The critical roles of defect sites and oxygen-containing groups in enhancing electrochemical performances of CNT-based electrodes have been proved by several fundamental research works [174, 175].

The influence of metallic impurities upon the electrochemistry of CNTs was also articulated by Compton and co-workers. They demonstrated that iron-based impurities within carbon nanotubes are responsible for the "electrocatalytic" oxidation of hydrazine and glucose [102, 176] and the reduction of hydrogen peroxide [177]. They also demonstrated that copper nanoparticle impurities within CNTs cause the "electrocatalysis" of halothane and glucose [178, 179]. Further, it has also been demonstrated that residual catalyst nanoparticles that are encapsulated within the CNT graphene lattice might still be chemically accessible and could participate in the redox chemistry of biomarkers through

the intercalation of molecules within the CNT lattice as deep as 12 nm [180]. An uncontrollable amount of impurities in the CNT samples is evidently not desirable. However, from a broader perspective it is possible to direct the controllable decorating of CNTs with metallic nanoparticles and use their electrocatalytic properties in a controllable way for enhancing the performance of sensors and energy-storage devices [181, 182].

1.5.2 Fullerenes

The unique structure of C_{60} has a distinct lack of edge plane like- sites/defects [183, 184] consequently the origin of the reported electrocatalysis is quite curious. The elegant work by Compton and co-workers [185] has clearly indicated that the origin of the electro-catalytic response observed at C_{60} modified carbon electrodes as reported by Tan, Bond and co-workers for the electrocatalytic detection of cysteine [161] is un-ambiguously due to graphite impurities in C_{60} [185]. Recently, the electrocatalytic detection of adenine and guanine [163], nandrolone [164], adenosine and guanosine [165] and most recently, salbutamol [186] and the determination of dopamine in the presence of ascorbic acid [187] have been reported using C_{60} – modified electrodes. The authors of these reports suggested that the observed electrocatalytic activity is due to the partially reduced conductive C_{60} film rather than graphite impurities. The reduction of C_{60} films in aqueous media is in fact the electrochemically reversible reduction of adventitious $C_{60}O_n$, with subsequent rapid loss of "O_2^-" in an irreversible chemical step. There is no evidence that C_{60} itself is reduced within the potential window of aqueous electrolytes. To this end, in the case of the target analyte nandrolone, Goyal and co-workers [188] have carefully examined the effect of metallic impurities in their C_{60} and they found that the removal of embedded metals from fullerene shifts the peak potential of nandrolone to more positive potentials and peak current decreases. Thus, the untreated fullerene modified electrode exhibits enhanced catalytic effect as compared to acid purified and super-purified C_{60} modified electrodes. Although, the origin of the electrocatalysis has always been attributed due to partially reduced conductive C_{60} film but recent reports have indicated that this is not the only reason. The presence of graphite impurities [189], metal impurities [102] and the pretreatment employed [190] may all be the origin or contribute significantly to the observed electrocatalysis at C_{60} – modified electrodes depending on the experimental parameters, chosen not only during the electrochemical measurements, but also during the synthesis of fullerenes. It is likely that other over-looked parameters may also contribute to the electrocatalytic effect of C_{60}

modified electrodes and thus it is recommended that control experiments should be performed before electrocatalysis of C_{60} modified electrodes is claimed. Thus, the curiosity of better understanding of electrocatalytic activity and the recognized demand for the development of highly sensitive and selective sensors has stimulated us to exploit fullerenes and carbon nanotubes as electrode surface modifiers for the determination of very low concentration of biomolecules and drugs in human body fluids.

1.6 TYPICAL STRUCTURE INFORMATION ABOUT NANO-MATERIALS USED IN THE THESIS

Recent advancement in sensing technologies based on nanomaterials has resulted by the development of several novel sensor devices with their challenging applications. The unique properties of carbon nanomaterials offer excellent prospects for interfacing analytical recognition events with electronic signal transduction and for designing new sensing devices. Carbon nanomaterials are very well suited for chemical sensor applications because their physical properties often vary considerably in response to the changes of chemical environment. Owing to these specifics the most common carbon nanomaterials, fullerenes and carbon nanaotubes are used for sensors applications in the present investigation. A summarized description regarding the structure of fullerenes and carbon nanaotubes and their well known characteristics are introduced in forthcoming paragraphs.

The discovery of an important allotrope of carbon referred as buckminsterfullerene or fullerene – C_{60} was a seminal event in the genesis of nanotechnology. The first fullerene was discovered in 1985 by Richard Smalley and Robert Curt from Rice University, and Harold (then of the University of Sussex, now of Florida University) for which they were awarded the 1996 Nobel Prize in Chemistry. This triggered enormous interest among the scientific community. The exploration of this new molecular form of carbon resulted in remarkable progress in the field and has got immense scope in nanoscience and technology.

Fullerene – C_{60} is a molecular crystal that contains sixty carbon atoms in a network of sp^2 arranged exactly as of football [191]. Each carbon atom is bonded to three others and is sp^2 hybridized. C_{60} is not "superaromatic" as it tends to avoid double bonds in the pentagonal rings which results poor electron delocalization. As a result, C_{60} behaves like an electron deficient alkenes and reacts readily with electron rich species. The geodesic and electronic bonding factors in C_{60} structure account for the stability of the molecule. These are zero dimensional and their diameter varies between 7-15 Å [192]. The structure of C_{60} is completely different to that of typical sp^2 carbon structures and cannot be discussed in terms

of the usual key structural factors. **Fig. 1.7** depicts the structure of C_{60} which is a truncated icosahedral structure with a polygon of 60 vertices and 32 faces, and 12 of which are pentagonal and 20 are hexagonal [193].

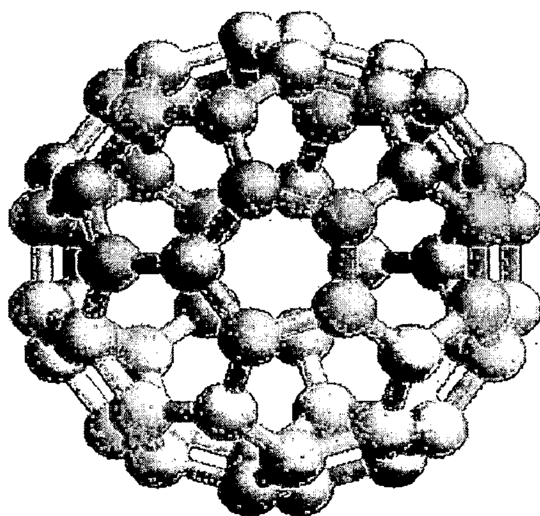


Fig. 1.7 The diagrammatic depiction of structure of C_{60}.

Similar to discovery of C_{60} a major breakthrough came in 1991 when Ijiyama of NEC Japan announced the synthesis of carbon nanotubes (CNTs). This is another important allotrope of carbon which has attracted the attention of many scientists worldwide. CNTs are electrochemically inert and do not exhibit voltammteric response in the potential window commonly used. These are conductive and stable in harsh chemical environment [194]. Since carbon nanotubes were discovered, the past decade witnessed significant progress in carbon nanotube synthesis as well as the investigations on their electrical, mechanical, optical and chemical properties [195, 196].

The structure of carbon nanotubes can be visualized as the cylindrical roll-up of one or more flat graphene sheets containing carbon atoms in a honeycomb arrangement [197]. The atomically monolayered nanotube surface contains sp^2 hybridized carbon atoms. Three out of the four outer-shell electrons of these carbons participate in bonding with neighbor carbons while the fourth electron is in a *p* orbital perpendicular to the hexagonal lattice. There are three basic methods for synthesis of nanotubes; electrical arch discharge, laser ablation (laser vaporization) and chemical vapor deposition (CVD) [198, 199]. CNTs are typically grown from nanosized metallic particles in the presence of a carbon source at temperatures exceeding 600 °C [200]. Depending on the nature and size of the catalyst as well as the temperature, carbon source, and a variety of processing conditions, nanotubes

grow off the metallic nanoparticles as single- or multi-walled carbon nanotubes. CNTs are closed structures that present two well defined regions with clearly different properties, the tube and the cap, which is half-fullerene-like molecule with topological defects that in this case are mainly pentagons [201, 202]. Basically, there are two groups of carbon nanotubes, multi walled (MWNTs) and single walled (SWNTs) carbon nanotubes. As shown in **Fig. 1.8** MWNTs can be visualized as concentric and closed graphite tubules with multiple layers of graphite sheets that define a hole typically from 2 to 100 nm separated by a distance of approximately 0.34 nm whereas, SWNTs consist of a single graphite sheet rolled seamlessly defining a cylinder of 0.4 – 3 nm diameter [203].

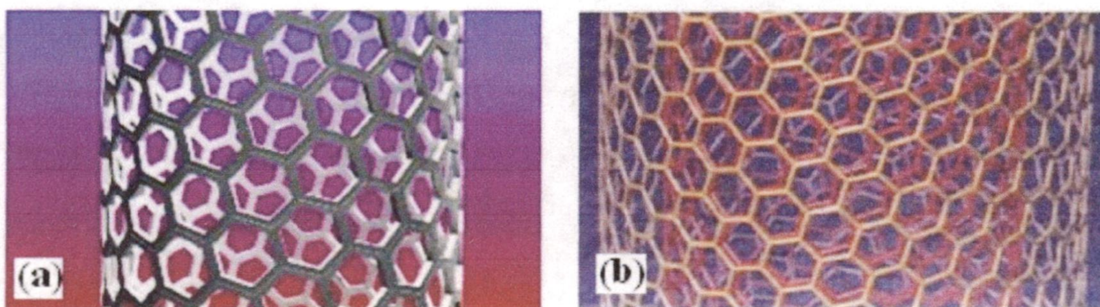


Fig. 1.8 Representation of an individual helical of (a) SWNT and (b) MWNT.

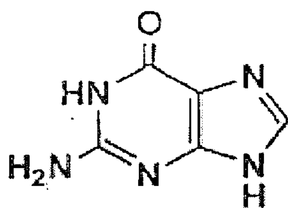
1.7 COMPOUNDS OF INTEREST

Biological systems and biomoecules are the foundation of all life on earth. Pharmaceutical drugs are an omnipresent factor of modern society having connections to the economy, pharmacotherapy, substance abuse, drug enforcement and crime. Hence, the science of understanding how to determine the actual and altered concentration of biomolecules and drugs in human body is increasingly important since; their altered concentration is indication of many diseases and doping cases. Doping is fundamentally contrary to the spirit of competitive games. A number of scientific and business areas are dedicated to the analysis and control of drug-like substances to discourage the illicit use of performance enhancing agents. Therefore, the quantitative determination of drugs or doping agents and biomolecules is portentous issue to improve human health, safety and quality of life. Hence, owing to these imperative facts studies have been carried out in the present dissertation that deals with the electro-oxidation and reduction of some important

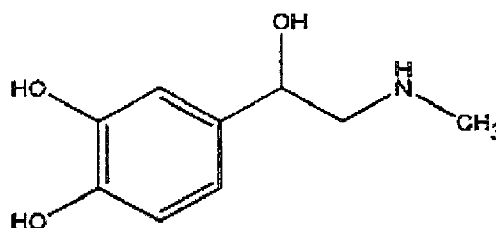
biomolecules and drugs or doping agents for their determination in human body fluids as well as pharmaceutical formulation.

Nucleic acids are found in all organisms with a variety of essential functions which store all information of the life. Guanine is highly susceptible to oxidative stress in the genomic DNA due to having the lowest oxidation potential hence; guanine play a key role in the oxidation of DNA by various types of oxidants and free radicals [204]. 8-Hydroxyguanine is one of the prominent lesions generated during DNA damage by oxidative processes and most extensively investigated due to its miscoding properties and potential role in mutagenesis, carcinogenesis and aging [205, 206]. A quantitatively important alteration occurring during oxidative damage to DNA consists of oxidation of guanine residues into 8-hydroxyguanine. Hence, guanine and 8-hydroxyguanine were choosed as target molecules and endeavor has been made to determine their relative concentrations in oxidatively damaged calf thymus DNA sample.

Catecholamines are produced by sympathetic nervous system activation and act as hormones and neurotransmitter to monitor heart rate, brain muscles activity, glycogenolysis, fatty acid mobilization and body temperature [207, 208]. Studies show that changes of their concentration in nervous tissues and body fluids are diagnostic symptoms of several diseases. The amount of catecholamines present in blood, plasma or serum is considered as a diagnostic aid to monitor therapeutic administration or to identify the causative agent in potential poisoning victims [209]. Hence, the quantitative determination of catecholamines is quite helpful for developing nerve physiology, clinical diagnosis of some diseases and controlling medicine in pharmacological research. The release of catecholamines in human system depends on smoking and exercise because these stimulants activates the sympathetic nervous system acting via splanchnic nerves to the adrenal medulla and stimulates the release of catecholamines into the blood stream [210, 211]. Therefore, blood plasma and urine samples of smokers and athletes are analyzed in the present thesis to determine catecholamines – epinephrine and norepinephrine concentrations.

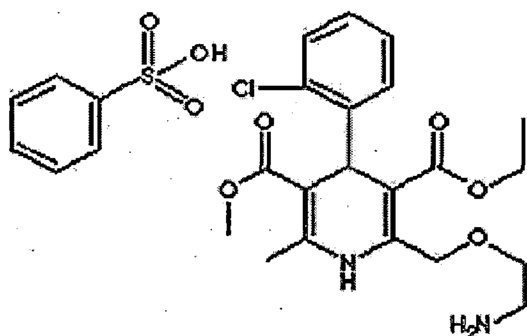


Guanine

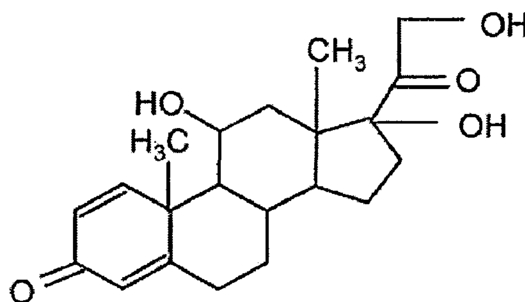


Epinephrine

Amlodipine is a third-generation dihydropyridine calcium antagonist which is used alone or in combination with other medications for treating high blood pressure, certain types of vasospastic angina, hypertension, cardiac arrhythmias, and coronary heart failure. Hence, in view of its importance in clinical cases studies have been carried out to determine its concentration in urine and plasma samples of angina patients undergoing treatment with amlodipine. Synthetic corticosteroids – prednisone and prednisolone have important physiological activities such as anti- inflammatory and anti-stress action and regarded as the most effective treatment for topical diseases [212, 213]. Both drugs also have the potential positive effects on sports performance. In human system prednisone is converted to the bioactive moiety prednisolone, via reduction of the 11-oxo group by 11 β-hydroxydehydrogenase liver enzyme [214]. In view of their metabolic relation and the clinical importance and increased abuse of prednisolone and prednisone by athletes, it is considered desirable to analyze their concentrations simultaneously in body fluids as well as in pharmaceutical formulations. Betamethasone has been extensively used in prenatal medicine to accelerate advance lung maturation in fetus of pregnant woman at risk of premature delivery [215]. Being a glucocorticosteroid, betamethasone is highly lipophilic and can readily cross the placenta thus, during the whole course of pregnancy the level of circulating drug is comparatively high in mother's body fluids. Hence, for diagnostic purposes it is quite helpful to detect its concentration in blood plasma or urine samples of pregnant women. Therefore, it is considered quite worthy to determine betamethasone concentration in these samples for treatment and better understanding of premature delivery cases.



Amlodipine Besylate



Prednisolone

The abuse of anabolic androgenic steroids (AASs) at supratherapeutic doses is a problem not only in the world of sports, but also among non athletes using AASs to improve physical appearance and to become more bold and courageous [216]. Nandrolone is an anabolic androgenic steroid banned in sports by the International Olympic Committee and World Anti-Doping Agency as it is extensively used by bodybuilders and athletes for the purpose of enhancing athletic performance [217]. Clinically, it is used in the treatment of anemia, neoplasia including breast cancer, rebuilding of muscles after debilitating disease and treatment of osteoporosis in postmenopausal women [218, 219]. In view of the clinical importance and increased abuse of nandrolone by athletes, it was considered worthwhile to analyse its concentration in body fluids as well as in pharmaceutical formulations.

Adenine is one of the two purine nucleobases and 2'-deoxyadenosine (2'-dAdo) is one of the purine 2'-deoxyribonucleosides present in deoxyribonucleic acid (DNA) and therefore, both are essential molecules of life and evolution. 2'-dAdo is a carbohydrate derivative of adenine and the conversion of 2'-dAdo to adenine represent a protective device to control the plasma level of 2'-dAdo when the activity of adenosine deaminase (ADA) is inhibited [220]. In the case of hepatocellular carcinoma, the level of adenine has been found to increase considerably [221]. Thus, the simultaneous determination of 2'-dAdo and adenine is the subject of considerable interest especially in human body fluids particularly urine in case of carcinoma. Thus, keeping in consideration the importance of adenine concentration in body fluids studies have been performed in the thesis to determine its concentration in urine sample of carcinoma patient.

1.8 SUBJECT MATTER OF THE THESIS

It is a challenging task to determine the concentration of physiologically important compounds in human body fluids owing to the dependence of toxicological, medical and ecochemical conclusion on the reliability of analysis. The very low concentration of biomolecules is an imperative difficulty to determine their level in body fluids. However, during the condition of pathophysiologic dysfunction and malfunctioning of body systems, the normal metabolism get altered, and lead to increase or decrease in the concentration of biomolecules in body fluids. The amount of biomolecules in the urine and plasma samples is considered as the marker of some diseases and, hence, it is of diagnostic value to determine their altered level. The use of performance-enhancing drugs continues to be one

of the most vexatious problems as it is detrimental to the reputation of competitive games. It is, therefore, mandatory for the accredited laboratories to develop an effective method which aims at the determination of doping agents. The quantitative determination of drugs is vital for pharmaceutical chemistry and clinical purposes. Due to expanding stipulate for the methodological development, an endeavor has been made in the present dissertation to develop simple, selective and sensitive electrochemical sensors using carbon nanomaterials for the determination of biomolecules, drugs or doping agents in human body fluids. For better understanding and clarity the results of the investigation have been organized in the dissertation as follows:

Chapter 1 – GENERAL INTRODUCTION

Chapter 2 – VOLTAMMETRIC SENSOR FOR THE DETERMINATION OF OXIDATIVE DNA DAMAGE IN CALF THYMUS DNA

Chapter 3 – DETERMINATION OF CATECHOLAMINES AT MWNT/EPPGE

Chapter 4 – DETERMINATION OF SOME IMPORTANT DRUGS/DOPING AGENTS AT SWNT/EPPGE

Chapter 5 – DETERMINATION OF DRUGS AND BIOMOLECULES AT C_{60} MODIFIED ELECTRODES

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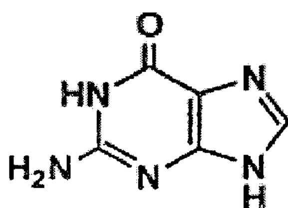
Chapter 2

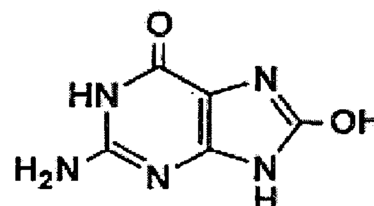
VOLTAMMETRIC SENSOR FOR THE DETERMINATION OF OXIDATIVE DNA DAMAGE IN CALF THYMUS DNA

2.1 INTRODUCTION

Nucleic acids are found in all organisms with a variety of essential functions. These important compounds store all information of life; constantly assaulted by oxidative stress caused from numerous endogenous metabolic processes as well as from exposure to environmental and dietary oxidants [1-3]. "Oxidative stress" is an increased generation or decreased elimination of reactive oxygen species which attack nucleic acids and generate oxidative lesions [4]. The continuous formation of hydrogen peroxide and superoxide anions is occurred during normal cell metabolism and these ions produce powerful oxidant hydroxyl radicals that attack cellular constituents such as proteins, nucleic acids and membrane phospholipids. Guanine is highly susceptible to oxidative stress in the genomic DNA due to having the lowest oxidation potential hence; guanine play a key role in the oxidation of DNA by various types of oxidants and free radicals [5]. 8-Hydroxyguanine is one of the prominent lesions generated during DNA damage by oxidative processes and most extensively investigated due to its miscoding properties and potential role in mutagenesis, carcinogenesis and aging [6, 7]. A quantitatively important alteration occurring during oxidative damage to DNA consists of oxidation of guanine residues into 8-hydroxyguanine. Literature survey reveals that there is very scarce information available regarding the simultaneous determination of guanine and 8-hydroxyguanine in oxidatively damaged DNA samples. Although, both compounds are determined individually by many methods such as chemoluminescence, voltammetry, mass spectrometry and high pressure liquid chromatography, simultaneous determination has been attempted only by isotope dilution mass spectrometry [8-12]. As, individual determination of 8-hydroxyguanine guanine or guanine can give misleading information about the extent of DNA damage hence, in the present paper an attempt has been made to simultaneously determine the relative concentrations of guanine and 8-hydroxyguanine in oxidatively damaged calf thymus DNA sample.



Guanine



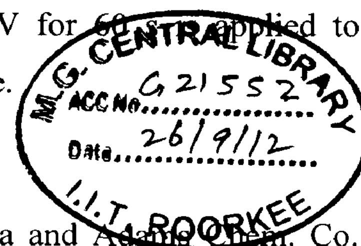
8-Hydroxyguanine

Isotope dilution mass spectrometry required isotopically labelled oxidized bases as internal standards to compensate for any loss of material in the work up and for chromatographic methods the isolation of matrix components as well as derivitization steps are frequently needed [13, 14]. Levels of several oxidized bases especially 8- hydroxyguanine is artifactually increased during derivatization of hydrolyzed DNA at high temperatures, leading to an overestimation of oxidative DNA damage. These extra precautionary steps are tedious, time consuming and require expensive instrumentation. Voltammetric methods employing nanomaterial modified electrodes have drawn extensive attention to determine various biomolecules due to their high sensitivity, reproducibility, stability and fast response [15-17]. The main advantages of voltammetric devices are their fast response, low-cost, small dimensions, simple design and low power requirements. Many voltammetric methods have been developed for the simultaneous determination of guanine and adenine using different solid electrodes such as carbon, silver, gold and diamond [18-21]. However, to the best of our knowledge, no voltammetric method has been developed to till date for the simultaneous determination of guanine and 8-hydroxyguanine in oxidatively damaged (acid hydrolyzed calf thymus) DNA samples although, this alteration is very closely related to mutagenesis, carcinogenesis and aging [22]. The reason for this may be, among others, merging of the peak of 8-hydroxyguanine with the background current due to its very low concentration in oxidatively damage DNA as compared to guanine. To overcome this serious problem sensitive voltammetric sensor has been developed in the present work using single-walled carbon nanotubes modified edge plane pyrolytic graphite electrode. The main advantage of the modified electrode is its selectivity, as it can detect very low concentration of 8-hydroxyguanine without affecting the other products of DNA damage. The results suggest that single-walled carbon nanotubes can effectively decrease the oxidation peak potential of guanine and 8-hydroxyguanine and greatly enhance their peak current. Good sensitivity, selectivity, reproducibility and stability of the modified electrode make it attractive for further development in the field of electrochemical sensors for monitoring DNA damage.

2.2 EXPERIMENTAL

2.2.1 Apparatus and voltammetric procedure

All voltammetric measurements were carried out using BAS (Bioanalytical Systems, West Lafayette, USA) CV-50W voltammetric analyzer. Single compartment electrochemical cell containing bare or modified edge plane pyrolytic graphite working electrode, a platinum wire counter electrode, and an Ag/AgCl (3 M NaCl) reference electrode (Model MF-2052 RB-5B) was employed for the voltammetric measurements. The surface morphology of bare and SWNT modified edge plane pyrolytic graphite electrodes was characterized using JEOL JSM-7400F field emission scanning electron microscopy (FE-SEM) instrument. Square-wave voltammetry was performed with the following parameters: square wave frequency (f): 15Hz, square wave amplitude (E_{SW}): 25 mV and potential step (E): 4mV. All potentials are referred to the Ag/AgCl reference electrode at an ambient temperature of 27 ± 2° C. The pH of buffer solutions was measured using a Century India Ltd. digital pH meter (Model CP-901). The voltammetric experiments were performed in 1.0 M phosphate buffer solution of different pH containing guanine and 8-hydroxyguanine at bare and modified electrodes in a suitable potential range. After a 2 s of the quiet period, the cyclic voltammograms were recorded at the scan rate of 20 mVs^{-1}. The modified electrode gives reproducible results for three consecutive runs in the same solution, however, before next sample a potential of – 100 mV for 60 s was applied to overcome the problem of adsorption of purines at electrode surface.



2.2.2 Chemicals and reagents

Guanine and 8-hydroxyguanine were obtained from Fluka and Adams Chem. Co. Illinois, USA respectively and used without further purification. Calf thymus DNA was purchased from Sisco Research Lab., Mumbai, India. Single- wall carbon nanotubes (SWNT) of purity > 98% were purchased from Bucky, USA. The stock solution (25×10^{-6} mol L^{-1}) of guanine was prepared in double distilled water and the stock solution of 8-hydroxyguanine (25×10^{-6} mol L^{-1}) was prepared in 2M Na_2CO_3 and then diluted with PBS (pH 7.2) to achieve the desired concentration. Other solvents and chemicals used were of analytical grade from Merck. Phosphate buffer solutions of different pH and ionic strength were prepared according to the method of Christian and Purdy by mixing standard solutions of Na_2HPO4 and NaH_2PO4 [23].

2.2.3 Preparation of DNA samples

Three milligrams of calf thymus DNA was hydrolyzed by addition of 1 mL of 60% formic acid and heated in water bath for 80 min. in an evacuated and sealed hydrolysis tube. Formic acid was used for DNA hydrolysis since it caused oxidative damage to DNA without causing any 'artifactual' oxidation [24]. Then, the pH of solution was adjusted to 7.2 with NaOH and solution was diluted to 10 ml using 1.0 M PBS of pH 7.2 in order to remove matrix complexity. Similarly different known concentrations of DNA were prepared and square wave voltammograms of the sample solutions were then recorded under optimized parameters. This gentle treatment lead to not only selective removal of purine bases by cleavage of purine glycoside bonds but also causes structural modifications of DNA nucleobases [25]. An important alteration consists of oxidation of guanine residues into 8-hydroxyguanines occurs when calf thymus DNA is exposed to a hydroxyl radical generating system [24].

2.2.4 Preparation of electrode

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 glass rod. Edge plane pyrolytic graphite piece ($1 \times 1 \times 3$ mm^3) was obtained from Pfizer Inc. New York, U.S.A. The piece was then inserted in glass tube carefully from the other open end of tube with the help of wire till 3/4th portion of it gets covered with epoxy resin to avoid any air pocketing between the tube and the 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 in order 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 electrode surface was then cleaned by rubbing it on a sand paper, followed by washing with distilled water before using it for experimental purposes.

2.2.5 Fabrication of modified pyrolytic graphite electrode

Modification of the electrode surface by nanotubes is of great importance including a wide spectrum of promising applications. Briefly, prior to the modification, the surface of the EPPGE was rubbed on an emery paper and washed with double distilled water, touched softly onto tissue paper. Suspension of SWNT (0.05 mg mL^{-1}) was prepar

dispersing 0.05 mg SWNT in 1.0 mL N, N-dimethylformamide (DMF) by ultrasonic agitation. The surface of the clean pyrolytic graphite electrode was then coated with 40 μL of this suspension and dried in a stream of hot air. The response of modified electrode was initially checked in a blank solution of phosphate buffer of pH 7.2. The absence of peak in entire potential region and very low background current indicate that the modified electrode can be safely used for further voltammetric studies. The surface of bare and modified electrodes was characterized using field emission scanning electron microscopy. A comparison of FE-SEM images of the two electrodes is presented in **Fig. 2.1**.

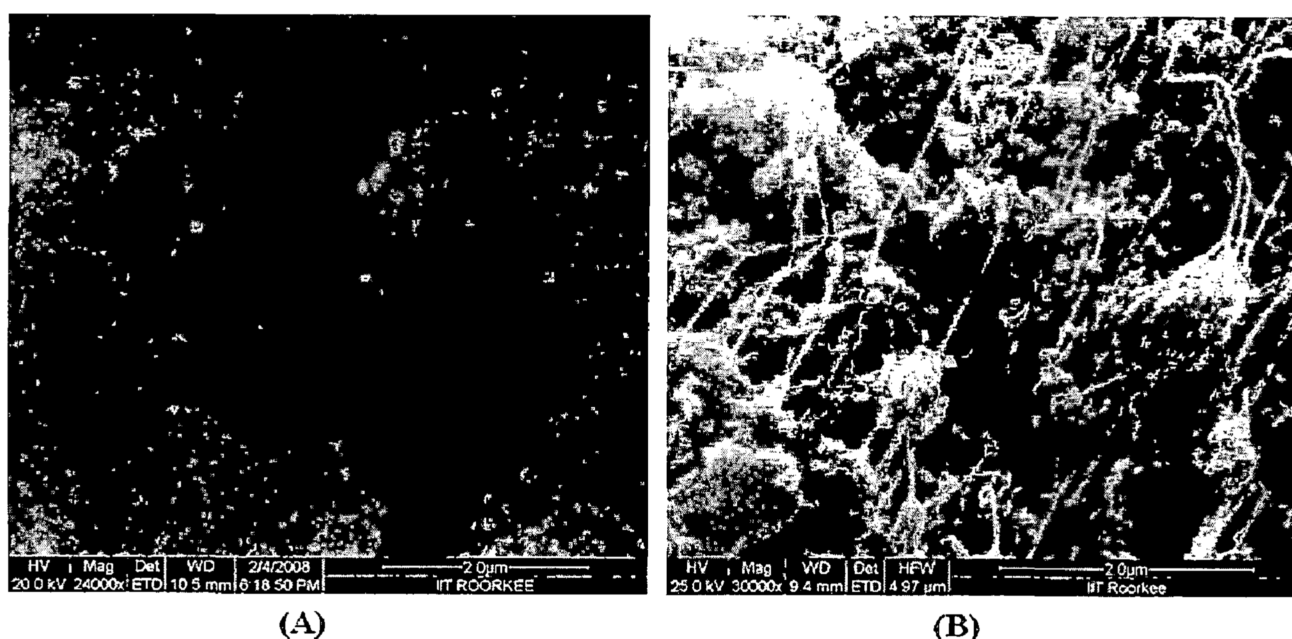


Fig. 2.1 Comparison of typical FE-SEM images of (A) bare and (B) SWNT modified edge plane pyrolytic graphite electrode.

2.2.6 Surface area

Since higher surface area of an electrode is expected to impart higher electrocatalytic activity resulting higher sensitivity [26, 27]. The effective surface areas of bare and SWNT modified EPPGE were obtained by recording cyclic voltammograms of 1mM $K_3Fe(CN)_6$ containing 0.1 M KCl at various scan rates. A well defined redox couple was observed due to the Fe^{+3}/ Fe^{+2} at both the electrodes. The peak-to-peak potentials separation was larger at bare electrode (~175 mV) than that on SWNT modified

electrode (~ 90 mV) and peak current was also high at modified electrode. Assuming semi-infinite planar diffusion for a reversible process:

$$i_p = 0.4463\ (F^3/RT)^{1/2} A\ n^{3/2}\ D_R^{1/2}\ C_0\ \nu^{1/2}$$

where F is Faraday's constant (96485 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 (Ampere), n = 1 for $K_3Fe(CN)_6$, T is the absolute temperature (298 K), $D_R = 7.6 \times 10^{-6}\ cm^2\ s^{-1}$, ν is scan rate (Vs^{-1}) and C_0 is the concentration of $K_3Fe(CN)_6$ in $molL^{-1}$.The surface areas of bare and modified electrodes were calculated from the slope of the i_p versus $\nu^{1/2}$ plot and found as 0.0489 and 0.1174 cm^2, respectively. The effective working area of modified electrode is 2.4 times larger than that of bare EPPGE. This indicates that among other major reasons such as; metal impurities in nanotubes and edge-plane-like defects which are present at the open ends of nanotubes, higher surface area also may be one of the reasons for improved electrocatalytic activity of modified electrode. However, thin-layer diffusion may also likely to operate in the modified electrode as suggested by Streeter [28].

2.2.7 Amount of SWNT suspension

Different amounts 5, 15, 20, 40, 60, 80 μL of SWNT suspension were casted on the surface of bare electrode, respectively. The experiment results indicate that when the modifying amount was too low or too high, the response of guanine and 8-hydroxyguanine and the stability of the modified electrode were not good. The optimum voltammetric response of guanine and 8-hydroxyguanine was observed using 40 μL of SWNT suspension. These observations show that too low amount cannot completely cover the surface of bare electrode, and too high amount makes uneven distribution of nanoparticles on the electrode surface which results in the congregation of nanoparticles. Hence, 40 μL aliquot of this suspension was selected as the optimum amount for modification for further studies.

2.3 RESULTS AND DISCUSSION

2.3.1 Comparison of bare EPPGE and SWNT/EPPGE

Firstly, square wave voltammogram of blank supporting electrolyte was recorded using SWNT/EPPGE at optimized parameters and conditions. It was found that no peak appeared in the entire potential region and the background current of modified electrode was significantly low. Then square wave voltammograms (SWVs) of mixture containing guanine and 8-hydroxyguanine were recorded utilizing bare and SWNT modified EPPGE in

phosphate buffer solution of pH 7.2 at square wave frequency of 15 Hz. **Fig. 2.2** clearly shows that at modified electrode guanine and 8-hydroxyguanine oxidized in two well-defined peaks located at ~ 508 and ~ 312 mV, respectively. However, on the bare EPPGE two broad peaks appeared at ~ 595 and ~ 401 mV with very small oxidation peak currents corresponding to the oxidation of guanine and 8-hydroxyguanine, respectively. These results clearly indicate that using modified electrode the shift (ΔE_p) of ~ 88 mV in peak potentials was observed both for guanine and 8-hydroxyguanine along with marked enhancement in peak current. The significant improvement in voltammetric response of both compounds strongly indicates that SWNT modified EPPGE exhibits strong electrocatalytic activity towards the oxidation of guanine and 8-hydroxyguanine due to fast electron transfer process [29, 30]. The origin of electro catalytic properties of nanotubes has been assigned to the embedded metal impurities in CNT samples [31, 32] and edge-plane-like defects which are present at the open ends of nanotubes [33].

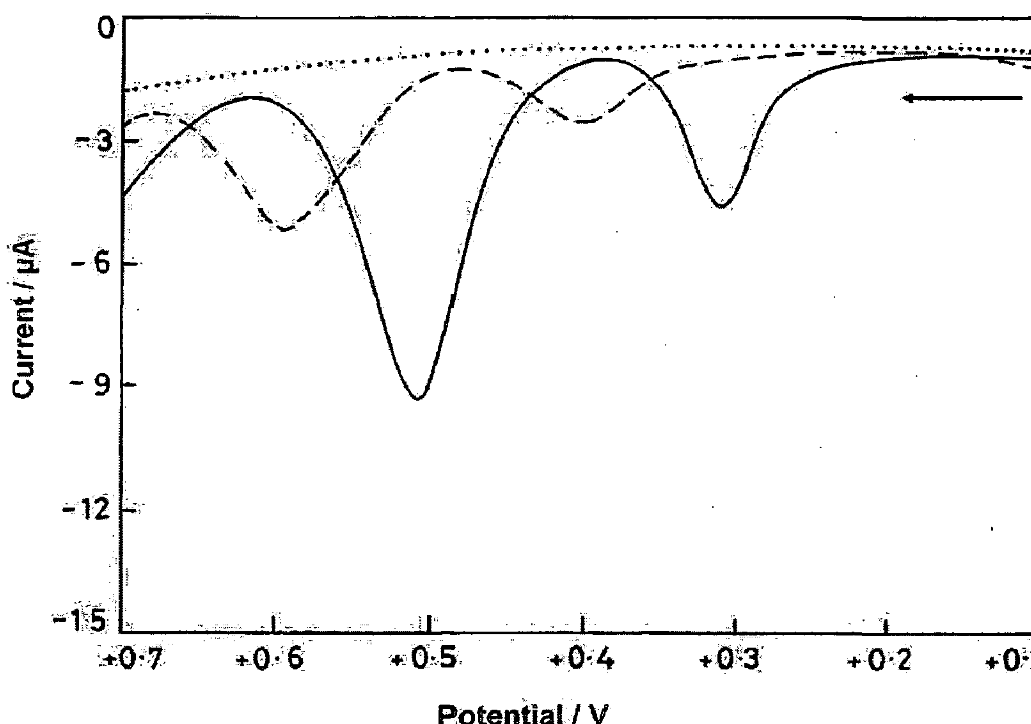


Fig. 2.2 Square-wave voltammograms of binary mixture contains 1.5 μM guanine and 2.5 nM 8-hydroxyguanine at (a) bare edge plane PGE (- - - -), (b) SWNT modified EPPGE (—) and dotted line is voltammogram of blank PBS using SWNT/EPPGE at pH 7.2.

2.3.2 Cyclic voltammetry

Cyclic voltammetric measurements were performed after deaeration of homogenous solutions of guanine and 8-hydroxyguanine with N_2 for at least 10 min. Cyclic voltammograms of binary mixture of guanine and 8-hydroxyguanine were recorded in phosphate buffer solution of pH 7.2 using bare and modified EPPGEs at scan rate of 20 mV/s. **Fig. 2.3** indicates that at bare EPPGE the oxidation of guanine and 8-hydroxyguanine occur with broad peaks at ~ 640 and ~ 452 mV, respectively. Since, it is practically impossible to deduce any quantitative information from the broad voltammetric peak hence, voltammograms were again recorded at SWNT modified EPPGE.

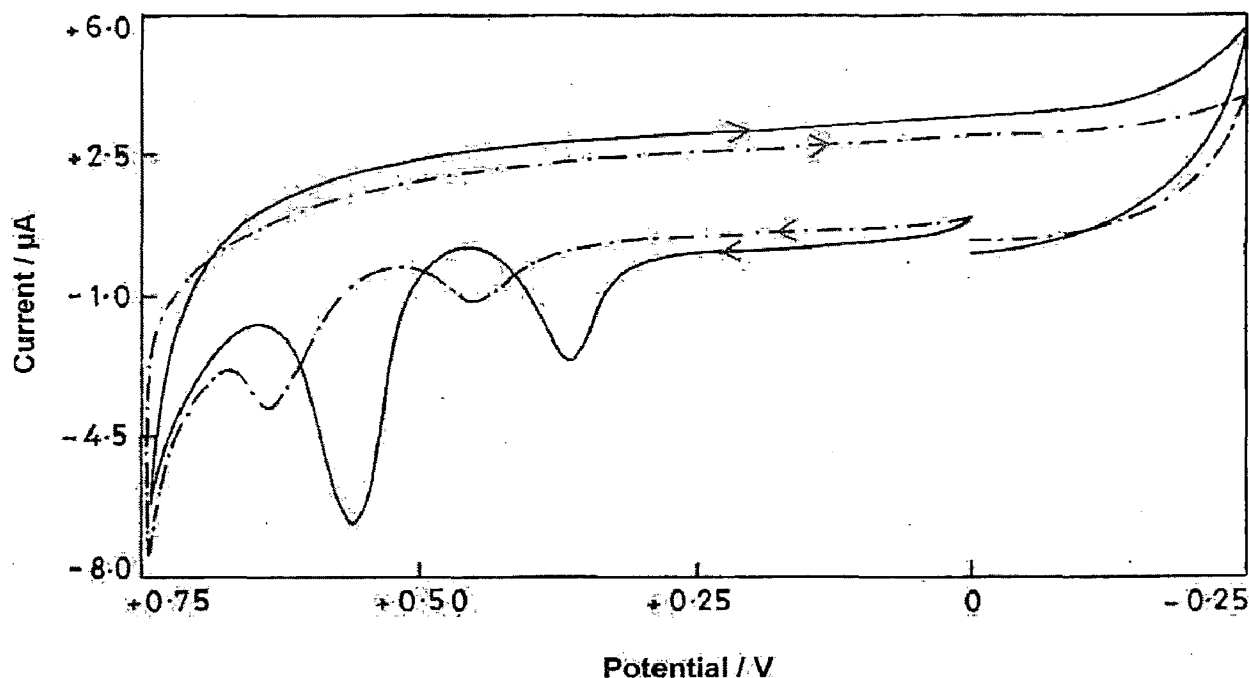


Fig. 2.3 Cyclic voltammograms of homogeneous solution of guanine and 8-hydroxyguanine in PBS of pH 7.2 using (a) bare edge plane PGE (-·-·-·) and (b) SWNT modified EPPGE (—) at scan rate of 20 mV/s.

After the modification, under the identical conditions the peak potential shifted negatively and oxidation occurred with well defined peaks at ~ 556 and ~ 360 mV for guanine and 8-hydroxyguanine, respectively along with substantial increases in peak current. The high sensitivity of the modified electrode was proved to be mainly attributed to the catalytic function of the nanotubes and low background current at the modified

electrode. Cyclic voltammograms show that only anodic peaks were observed for guanine and 8-hydroxyguanine at both bare and modified EPPGEs. Absence of peak in reverse scan indicates that both the compounds are oxidized irreversibly using graphite electrode at optimized scan rate. Since square wave voltammetry has a much higher current sensitivity and better resolution than cyclic voltammetry, it was used for the determination of guanine and 8-hydroxyguanine in real samples.

2.3.3 Electrochemical characteristics of guanine

An important factor which affects redox potential of analytes is the pH of supporting electrolyte. The effect of pH on the electrode response and the oxidation potential was determined by square wave voltammetry in the solution containing guanine (1.5 μM) at square wave frequency of 15 Hz. A higher pH value made the anodic peak potential shift negatively. The plot of the peak potential versus pH shows linearity in the pH range 2.4 to 10.4 with a slope of ~ 58 $mVpH^{-1}$. This implies that the ratio of the participated protons to the transferred electrons through the modified electrode surface is 1:1 [34]. The relationship between the oxidation potential (E_p) and pH value can be expressed by the following equation having correlation coefficient 0.997.

$$E_p \text{ (pH 2.4 – 10.4)} = [931.0 - 57.50 \text{ pH}] \text{ versus Ag / AgCl}$$

Square wave frequency is also an important parameter hence, the effect of square wave frequency on the peak current and peak potential of guanine was studied at pH 7.2. It was observed that as the square wave frequency increased the oxidation peak current of guanine increased. The peak current was directly proportional to the square wave frequency over the range 5 to 200 Hz, which suggested that the electrooxidation of guanine using modified electrode is an adsorption-controlled process [35]. The dependence of peak current on square wave frequency at SWNT/EPPGE can be expressed by the relation:

$$i_p\ (\mu A) = 0.058 f\ (Hz) + 7.562$$

with a correlation coefficient of 0.996. It was also observed that the peak potential shifted towards more positive potentials over the square wave frequency range 5 to 200 Hz and the

E_p versus log f plot was linear. The variation of peak potential with square wave frequency can be expressed by the equation:

$$E_p \text{ (mV)} = 196.6 \log f + 275.1$$

having correlation coefficient 0.998.

The oxidation peak current of different concentration of guanine was measured by square wave voltammetry using SWNT/EPPGE in PBS of pH 7.2 and then plotted against the concentration of guanine after background subtraction. The oxidation peak current was directly proportional to the concentration of guanine as shown in **inset (a)** of **Fig. 2.4 (A)**. The dependence of the peak current on the concentration of guanine is in a linear relationship and the linear regression equation is expressed as:

$$i_p \text{ (}\mu\text{A)} = 5.387 \text{ C (}\mu\text{M)} + 0.196$$

having correlation coefficient of 0.998. The limit of detection of guanine was calculated to be 0.05×10^{-9} mol L^{-1} and limit of quantification was found to be 0.17×10^{-9} mol L^{-1}.

2.3.4 Electrochemical characteristics of 8-hydroxyguanine

The effect of pH on the electrooxidation of 8-hydroxyguanine was explored by square wave voltammetry in the solution containing 2.5 nM 8-hydroxyguanine. The E_p versus pH graph shows that the oxidation peak potential of 8-hydroxyguanine shifted negatively with the increment of the solution pH, which indicated that protons were involved in the electrode reaction. The slope of ~ 52 $mVpH^{-1}$ reveals that the proportion of the electron and proton involved in the reactions is 1:1. A linear relationship was established between the oxidation peak potential and the solution pH with the linear regression equation as:

$$E_p \text{ (pH 2.4} - 10.4) = [682.1 - 51.79 \text{ pH}] \text{ versus Ag / AgCl}$$

with correlation coefficient 0.998.

In order to determine the effect of square wave frequency on the current response of 8-hydroxyguanine the square wave voltammograms of 8-hydroxyguanine were recorded at various square wave frequencies using SWNT/EPPGE in PBS of pH 7.2. The peak current showed a linear increase with the increase of square wave frequency in the range 5 to 200 Hz. These results indicate that the electrooxidation of 8-hydroxyguanine is an

adsorption controlled process [36]. The relationship between the oxidation peak current and square wave frequency was established with a linear regression equation as:

$$i_p\ (\mu A) = 0.052 f (Hz) + 2.87$$

having correlation coefficient of 0.992.

The increase in peak current was also accompanied by increase of peak potential with square wave frequency in the range 5 to 200 Hz. The oxidation peak potential increased gradually and the dependence of E_p with $\log f$ can be expressed as:

$$E_p\ (mV) = 167.3 \log f + 115.1$$

with correlation coefficient of 0.994.

Square wave voltmmograms of different concentrations of 8-hydroxyguanine were recorded in PBS of pH 7.2 using SWNT/EPPGE. It was found that the peak current increased with the increase in concentration of 8-hydroxyguanine as shown in **inset (b)** of **Fig. 2.4 (B)**. The calibration curve yielded a linear range from 0.03×10^{-9} to 7.5×10^{-9} mol L^{-1} with a linear regression equation of:

$$i_p\ (\mu A) = 1.514\ C\ (nM) + 0.205$$

having correlation coefficient of 0.996. The detection limit of 8-hydroxyguanine was calculated to be 0.01×10^{-9} mol L^{-1} and limit of quantification was found as 0.34×10^{-10} mol L^{-1}.

2.3.5 Simultaneous determination of guanine and 8-hydroxyguanine

The main aim of the present work is to simultaneously determine guanine and 8-hydroxyguanine hence, to verify the ability of the modified electrode to promote the voltammetric resolution of guanine and 8-hydroxyguanine, the square wave voltammetric response of the mixture of these species was determined utilizing SWNT/EPPGE in PBS of pH 7.2. Firstly, square wave voltammograms for increasing concentrations of guanine in the presence of a constant concentration of 8-hydroxyguanine at 2.0×10^{-9} mol L^{-1} were recorded. It can be seen clearly in **Fig. 2.4 (A)** that the peak current of guanine increased with increasing concentration without effecting the response of 8-hydroxyguanine. Similarly, the square wave voltammograms for increasing concentration of 8-hydroxyguanine by keeping the guanine concentration fixed at 0.5×10^{-6} mol L^{-1} were recorded. **Fig. 2.4 (B)** clearly shows that the voltammetric signal of 8-hydroxyguanine increased with the increasing concentration without any change in the response of guanine.

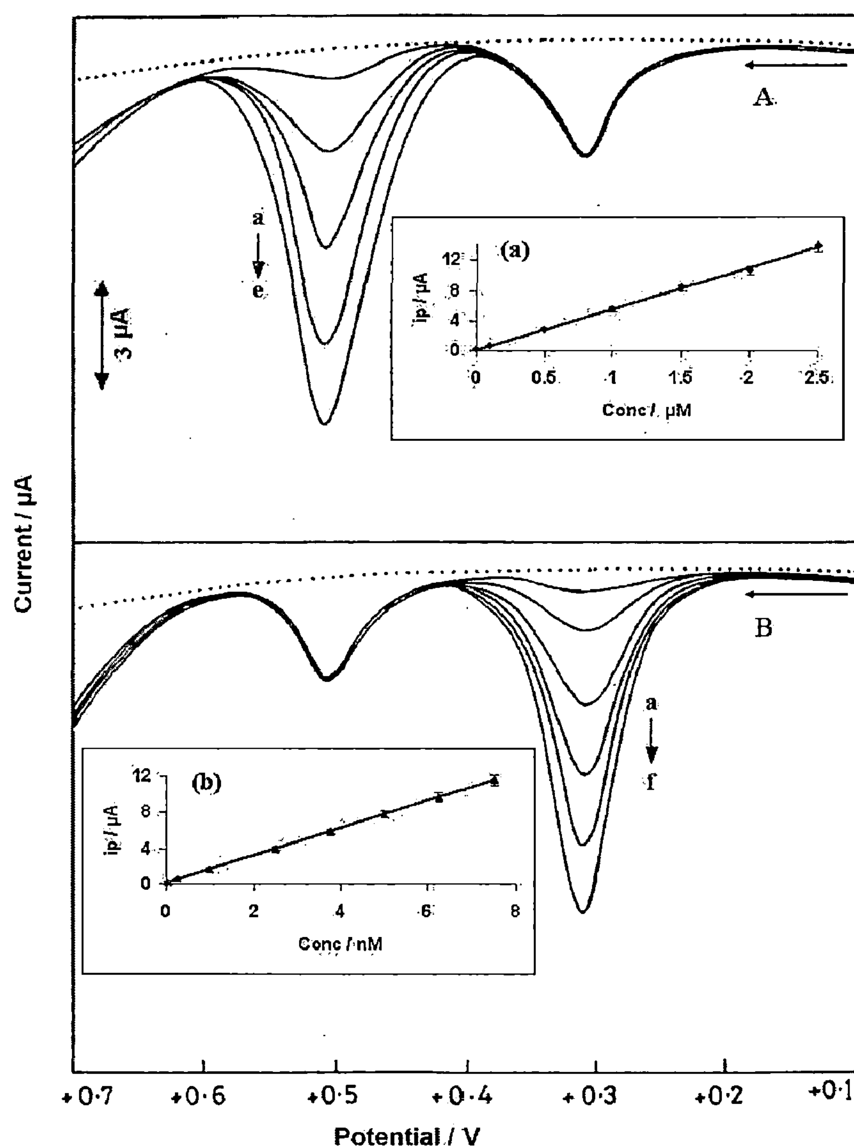


Fig. 2.4 Square wave voltammograms observed for (i) phosphate buffer solution (background) at SWNT/EPPGE (.....) and (ii) (A) increasing concentration of guanine at a fixed concentration of 8-hydroxyguanine; [8-hydroxyguanine] = 2.0 $\times 10^{-9}$ mol L^{-1}; [guanine]: a=0.1, b=0.5, c=1.0, d=1.5, e=2.0 µM and calibration curve for guanine [inset (a)], (B) various concentrations of 8-hydroxyguanine at a constant concentration of guanine; [guanine] = 0.5 $\times 10^{-6}$ mol L^{-1}; [8-hydroxyguanine]: a=0.25, b=1.00, c=2.50, d=3.75, e=5.00, f=6.25 nM and calibration curve for 8-hydroxyguanine [inset (b)] at pH 7.2.

Square wave voltammograms with different concentrations of both guanine and 8-hydroxyguanine in the same solution were also recorded. **Fig. 2.5** clearly indicates that when concentrations of both compounds increase simultaneously; both compounds exhibits oxidation peaks separately without interfering each other.

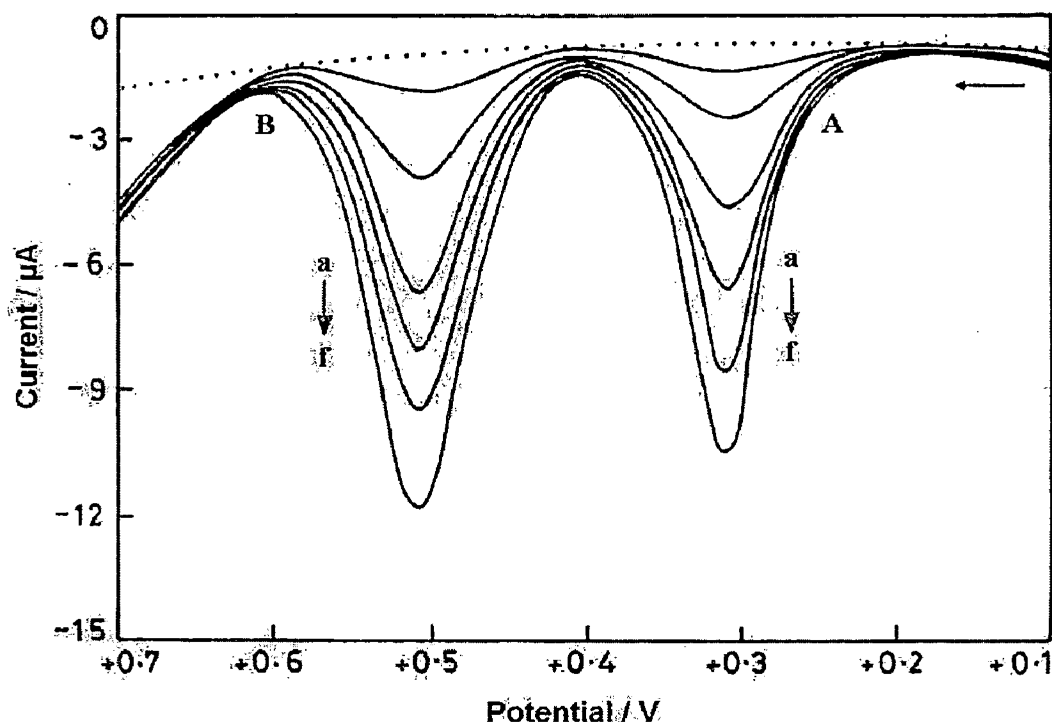


Fig. 2.5 Square wave voltammograms observed for (i) phosphate buffer solution (background) at SWNT/EPPGE (.....) and (ii) for the simultaneous determination of 8-hydroxyguanine and guanine with different concentrations (A) [8-hydroxyguanine]: a=0.25, b=1.00, c=2.50, d=3.75, e=5.00, f=6.25 nM and (B) [guanine]: a=0.10, b=0.50, c=1.00, d=1.25, e=1.50 f=2.00 µM at pH 7.2.

The above results indicated that the electrochemical signals of guanine and 8-hydroxyguanine were independent of each other at SWNT/EPPGE with the oxidation peak potential separation as ~ 0.196 V, which was enough to allow the simultaneous determination of these two compounds in a homogeneous solution. It is very interesting to note that the detection limit of the modified electrode towards 8-hydroxyguanine in the absence and presence of guanine is virtually the same, which indicates that the oxidation

processes of guanine and 8-hydroxyguanine are independent at the modified EPPGE and therefore, simultaneous determination of both species is easily possible without interfering each other. These observations promoted us to use modified electrode for the simultaneous determination of guanine and 8-hydroxyguanine in real samples.

2.3.6 Analysis of calf thymus DNA

8-Hydroxyguanine is one of many lesions generated in DNA damaging by oxidative processes. An important alteration consists of oxidation of guanine residues into 8-hydroxyguanines when calf thymus DNA is exposed to a hydroxyl radical generating system. Formic acid was used for DNA hydrolysis since it does not cause any artifacts. The SWNT/EPPGE has been utilized to detect oxidative DNA damage to calf thymus DNA samples. DNA sample has been prepared according to the above mentioned procedure and then square wave voltammograms of different concentrations of DNA sample were recorded under exactly same conditions and parameters which were used during calibration curve studies. Other purine and pyrimidine derivatives are also likely to be present in the matrix. Thymine, adenine and cytosine oxidize at higher potentials than guanine and 8-hydroxyguanine and thus do not interfere in eth determination. **Fig. 2.6** shows the square wave voltammograms of different concentrations of acid-hydrolyzed calf thymus DNA and consists of two oxidation peaks corresponding to the oxidation of guanine and 8-hydroxyguanine residues. The actual concentrations of both the compounds in DNA sample (a) 0.300 mg mL^{-1} were determined using calibration curves of both standards and found as 1.95 μM and 0.12 nM for guanine and 8-hydroxyguanine, respectively. The concentrations of guanine and 8-hydroxyguanine in DNA sample (b) 0.325 mg mL^{-1} were found as 2.11 μM and 0.14 nM respectively while the concentrations of both the compounds in DNA sample (c) 0.350 mg mL^{-1} were found as 2.27 μM and 0.16 nM for guanine and 8-hydroxyguanine, respectively. To reconfirm the results obtained recovery studies were carried out by adding the known concentrations of each standard in DNA samples of different concentrations.

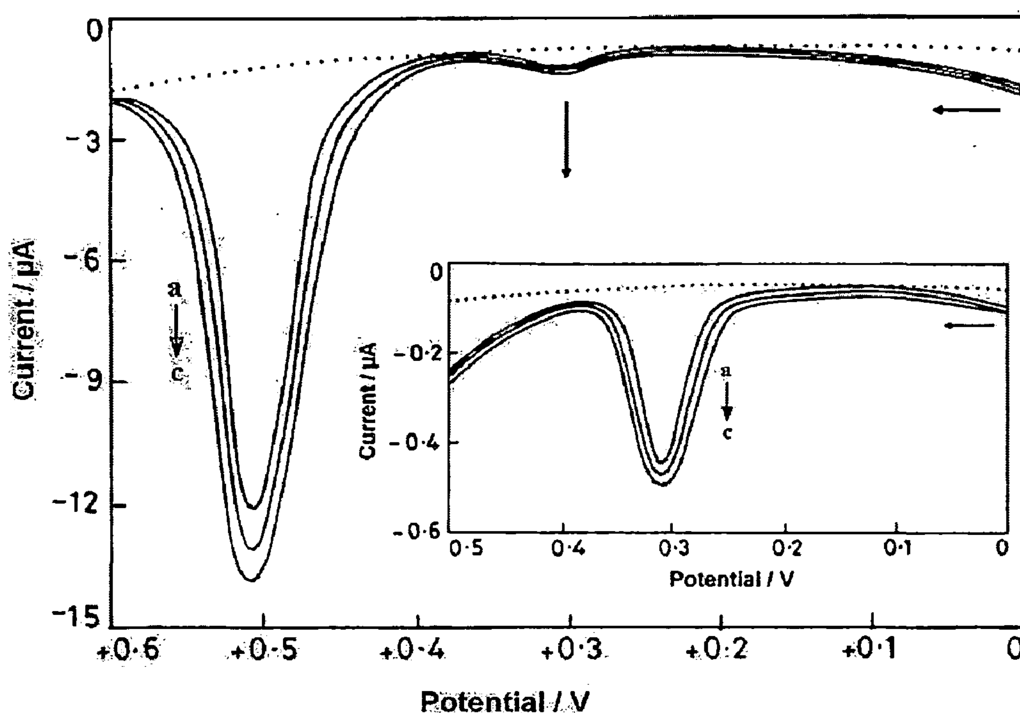


Fig. 2.6 Square wave voltammograms for the simultaneous determination of guanine and 8-hydroxyguanine in acid-hydrolyzed calf thymus DNA with increasing concentration from 0.300 to 0.350 mg mL^{-1} and dotted line is SWV of blank PBS using SWNT/EPPGE at pH 7.2. The inset shows the peaks of 8-hydroxyguanine.

2.3.7 Recovery

In order to evaluate the accuracy of the proposed method recovery experiments were performed using standard addition method. A known amount of each standard solution was added in successive aliquots to the cell containing DNA sample and then square wave voltammograms were recorded after each addition utilizing SWNT modified EPPGE. Three DNA samples of different concentrations were spiked with known amounts of standard guanine and 8-hydroxyguanine subsequently followed by recording their voltammograms. In all the cases two separate well-defined peaks were observed with E_p ~ 312 and ~ 502 mV corresponding to 8-hydroxyguanine and guanine, respectively. The concentration of both the compounds was calculated using proposed method and the results are listed in **Table 2.1**. The recoveries varied in the range from 99.23 to 101.08 % in the case of guanine and from 96.77 to 103.13 % in case of 8-hdroxyguanine. The recovery study indicates that the accuracy of the proposed voltammetric sensor is good and it can thus be recommended for monitoring DNA damage.

Table 2.1 Recovery results obtained for guanine and 8-hydroxyguanine in DNA samples of three different concentrations using SWNT/EPPGE

Guanine*			8-hydroxyguanine*		
Spiked (μM)	Detected (μM)	Recovery (%)	Spiked (nM)	Detected (nM)	Recovery (%)
DNA Sample					
0.300 mgmL^{-1}					
0.00	1.95	---	0.00	0.12	---
0.50	2.47	100.82	0.50	0.60	96.77
1.00	3.44	99.71	1.00	1.64	101.23
0.325 mgmL^{-1}					
0.00	2.11	---	0.00	0.14	---
0.50	2.59	99.23	0.50	0.66	103.13
1.00	3.64	100.83	1.00	1.62	98.78
0.350 mgmL^{-1}					
0.00	2.27	---	0.00	0.16	---
0.50	2.80	101.08	0.50	0.64	96.97
1.00	3.75	99.47	1.00	1.68	101.20

* The R.S.D value was < 1.8 % for guanine and < 2.2 % for 8-hydroxyguanine for $n = 5$

2.3.8 Electrode stability and reproducibility

It is not so important for the modified electrode to be stable for a prolonged time since the procedure of electrode preparation is easy and rapid. However, long-term stability of electrode has been checked by measuring the response from day by day. During the first five days the current response had no apparent change and in the next ten days the current response decreased about 4.8% of its initial response, and 16% after 15 days. These results indicate that modified electrode can be safely used for first 15 days after its preparation during storage at room temperature.

To characterize the reproducibility of the modified electrode repetitive measurements were carried out in a solution containing 2.5 μM of each guanine and 8-

hydroxyguanine. The results of 10 successive measurements showed a relative standard deviation of 2.8% and 2.2% for guanine and 8-hydroxyguanine, respectively. This indicates that the modified electrode shows excellent reproducibility towards the oxidation of both species. The voltammetric response of guanine and 8-hydroxyguanine at four independently modified electrodes based on the same bare electrode shows an acceptable reproducibility with a relative standard deviation of ±3.2%, indicates that the SWNT/EPPGE had an excellent reproducibility towards the simultaneous determination of both compounds. As the electrode fabrication is very easy and low cost, the modified electrode seems to be of great utility for making a voltammetric sensor to determine DNA damage.

2.4 CONCLUSIONS

Efficient and inexpensive tools are still required for better diagnose, prevention and treatment of many human diseases which originate from oxidative DNA damage. Veterinary and forensic medicine, and environmental testing also require rapid and accurate assays for DNA damage. Therefore, development of a simple, fast and reliable method for analysis of DNA damage products is of great importance. The proposed sensor has been used for the simultaneous determination of guanine and 8-hydroxyguanine in acid hydrolyzed DNA with satisfactory results. The SWNT/EPPGE showed great improvements in voltammetric response of guanine and 8-hydroxyguanine in terms of yields of large peak currents and lower peak potential as compared to bare EPPGE. Nanotubes provide synergic influence on the accurate electrochemical determination of guanine and 8-hydroxyguanine from a mixture having any one of the component (8-hydroxyguanine or guanine) in excess. Guanine and 8-hydroxyguanine coexisting in a homogeneous solution can be simultaneously determined using modified electrode and the separation of the oxidation peak potentials for guanine and 8-hydroxyguanine is 0.196 V, which is large enough for the simultaneous determination of two compounds in a homogeneous solution. Good sensitivity, selectivity, reproducibility and stability of the modified electrode made itself attractive for further development in the field of electrochemical sensors for nucleic acids.

Oxidative damage to DNA bases by GC-MS requires a derivatization procedure [14] which can cause 'artifactual' oxidation of some undamaged bases, leading to an overestimation of their oxidation products, including 8-hydroxyguanine. Therefore, using proposed method which requires no derivatization steps and hence eliminates possible 'artifactual' oxidation of DNA bases, level of guanine and 8-hydroxyguanine can be

measured in calf thymus DNA with high level of accuracy. It is concluded that proposed sensor meets all the essential conditions required to analyze DNA damage and also that the field of the voltammetric determination of the nucleic acids using nanomaterials modified graphite electrodes is very promising and important breakthroughs can be expected in the coming years.

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Chapter 3

DETERMINATION OF CATECHOLAMINES AT MWNT/EPPGE

3.1 INTRODUCTION

Electrode surface modification is a field of paramount importance in the modern electrochemistry especially due to the various application possibilities of modified electrodes. Carbon nanotubes (CNTs), which is currently in the forefront of materials research, play an important role in different areas including engineering, biology, chemistry, medicine, electronics and material science [1, 2]. It is well known that superior to the side walls of the CNTs, which are very similar to basal-plane graphite and show slow electron transfer rates, the open ends of the CNTs have excellent electrochemical properties [3]. Such an excellent electrochemical property of the open ends of the CNTs, e.g. fast electron transfer rate, is expected to be particularly attractive for electrochemical applications, especially for electrocatalysis. Due to their potential utility in fabricating highly-sensitive nanoscale electrochemical sensors by facilitating the electron transfer and enhancing the electrode conductivity, thus improving the analytical sensitivity and selectivity CNTs have attracted substantial interest in electrochemical determinations [4, 5]. These effects are attributed to the larger available surface area of the nanotubes modified electrodes, presence of metallic impurities in nanotubes, special electronic structure and the topological defects present on the tube surface [6-8]. Available literature indicates that two types of CNT materials are known, i.e., single-walled (SWNT) and multi-walled carbon nanotubes (MWNT) which consist of single and several graphite layers in the wall of a tube, respectively [9-11]. The lengths of these CNT are usually in the micrometer range with diameters from 0.4 to 3 nm for SWNT and from 1.4 to 100 nm for MWNT. The electrocatalytic properties of the CNTs are believed to be favorable for the oxidation and reduction of electroactive species towards cathodic and anodic direction, respectively alongwith the enhancement in peak current. Multi-walled carbon nanotubes have been widely used as novel electrode surface modifiers in electrochemical fields [12-15]. The subtle electronic properties suggest that MWNT have the ability to mediate electron transfer reactions with electroactive species in solution when used as an electrode surface modifier [16]. Recent studies have demonstrated improved electrochemical behavior of sterigmatocystin [12], brucine [13], promethazine [14] and urapidil [15] at MWNT modified glassy carbon, gold and carbon paste electrodes, respectively. In view of the excellent electrocatalytic properties of MWNT, in the present study multi-walled carbon nanotubes modified edge plane pyrolytic graphite electrode (MWNT/EPPGE) was examined to

determine catecholamines – epinephrine (EP) and norepinephrine (NE). The chapter is devided into two sections – first section deals with the individual determination of epinephrine while the second section describes the simultaneous determination of epinephrine and norepinephrine.

Catecholamines are produced by sympathetic nervous system activation and act as hormones and neurotransmitter to monitor heart rate, brain muscles activity, glycogenolysis, fatty acid mobilization and body temperature [17, 18]. Catecholamine drugs are used to treat hypertension, bronchial asthma, organic heart disease and also in cardiac surgery and myocardial infarction [19]. Epinephrine, also known as adrenaline (EP), is one of the important catecholamine, plays a central role during physical or mental stress and also stimulates a series of actions of the sympathetic nervous system (SNS) known as the "flight or fight response" [20]. It prepares the body for action in perceived emergency situations, boosting the supply of oxygen and energy-giving glucose to the brain and muscles [21]. It elevates the blood sugar level by increasing catalysis of glycogen to glucose in the liver, and at the same time begins the breakdown of lipids in fat cells [21]. These important actions of EP also make it a potent doping agent and hence, it is banned in competitive games by World Anti-Doping Agency [22, 23]. Clinically, EP has been utilized as a common emergency healthcare medicine such as a drug to treat cardiac arrest, dysrhythmias and as a bronchodilator for asthma [24]. It is also used to treat anaphylaxis and sepsis because of its suppressive effect on the immune system [25]. Studies show that changes of EP concentration in nervous tissues and body fluids are diagnostic symptoms of several diseases [26]. The amount of EP present in blood, plasma or serum is considered as a diagnostic aid to monitor therapeutic administration or to identify the causative agent in potential poisoning victims [27]. The quantitative determination of EP concentration is also quite helpful for developing nerve physiology, clinical diagnosis of some diseases and controlling medicine in pharmacological research [28]. Therefore, it is important to examine its electrochemical behavior and to develop a quantitative method for studying its concentration in body fluids.

Numerous electrochemical methods have been developed to determine EP due to its electroactive nature [29-40]. Most of these reported methods have two major problems in EP determination that reduce accuracy and sensitivity of the method. The first is that in natural environment epinephrine often exists together with high concentration of electroactive biomolecules like uric acid, dopamine, norepinephrine, ascorbic acid that

interfere with each other. The second problem of the reported methods is that the product of EP oxidation (epinephrinechrome) can easily transform into polymers, which block its further oxidation on the electrode surface. Hence, despite of considerable investigation, the preparation of a sensitive sensor with satisfactory selectivity and low detection limit with high sensitivity is still of great interest. In the present work, following the idea of searching new methods for EP detection and to overcome the above mentioned problems, the electrochemical reactivity of EP is examined by using edge plane pyrolytic graphite electrode modified with multi-walled carbon nanotubes (MWNT/EPPGE). To overcome the uric acid and ascorbic acid interference a specific potential region was selected by using MWNT/EPPGE for the determination of catecholamines where uric acid and ascorbic acid do not interfere with the determination. To regenerate the electrode surface time base technique has been utilized. The modified electrode surface was cleaned in 0.5 M phosphate buffer solution (PBS) by applying a potential of − 200 mV (for oxidation study) for 60 s. To the best of our knowledge no voltammetric method is available till date to analyze epinephrine concentration in plasma samples. Moreover, in most of the recently reported methods only recovery study has been reported for EP analysis in urine sample [34-37]. As recovery study just provides the information about the stability of the compound in body fluids, which is important in the case of drugs analysis, hence proved the stability of free epinephrine in body fluids. However usefulness of these reported methods could not be ascertained. Hence, considering the expanding demand, the developed protocol using MWNT/EPPGE has been implemented for the determination of EP in human urine as well as plasma samples.

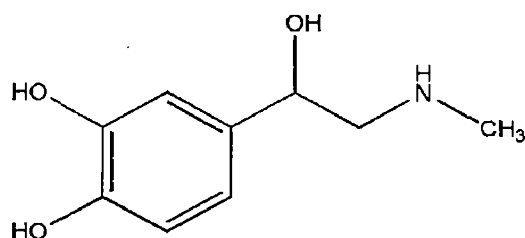
Simultaneous determination of catecholamines – epinephrine and norepinephrine is of great importance since both catecholamines have complementary actions in human body [41]. The biosynthesis of both the neurotransmitters in human system occurs from tyrosine which is produced in the liver from phenylalanine [42]. Tyrosine then transported into catecholamine-secreting neurons and adrenal medullary cells where it convert into dopamine and finally to epinephrine via norepinephrine through a series of reactions [42]. Epinephrine, known as the "fight or flight" hormone, energizes and speeds up the various systems within the body and plays an important role during the times of stress [43]. While norepinephrine (NE) increases the conversion of glycogen to glucose in the liver, helps in converting fats into fatty acids, and relaxing the bronchial muscles [44]. All these actions of norepinephrine are related to calm down of the body. Owing to the fact that EP is a

derivative of NE and both catecholamines have complementary actions in human body, it has been considered worthwhile to develop a selective and sensitive method for the simultaneous determination of epinephrine and norepinephrine in human body fluids. Several methods based on voltammtery have been developed for the individual determination of NE and its analogs EP [31, 33, 45, and 46]. But these reports have comparatively lesser importance to monitor physiological functions since; EP and NE have complementary actions in the body. Only single voltammteric method has been reported till date for the simultaneous determination of EP and NE however, analysis has not been carried out in human body fluids [38]. One of the reasons for this is due to oxidation of both the neurotransmitters at almost the same potential which results in overlapped voltammetric responses, and makes their discrimination very difficult in the same solution [47]. The carbon paste electrode reported for the simultaneous determination of epinephrine and norepinephrine having no real sample analysis although, the electrode modification procedure was enough complicated [38]. Hence, there is still an expanding demand for the development of a simple, selective and sensitive sensor that can resolve overlapped voltammetric signals of epinephrine and norepinephrine.

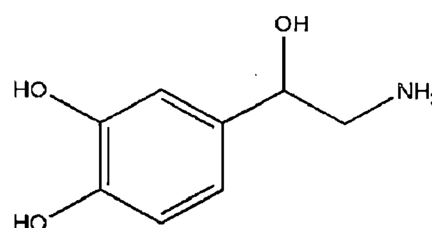
Literature survey reveals that the release of catecholamines in human system depends on smoking and exercise because these stimulants activates the sympathetic nervous system acting via splanchnic nerves to the adrenal medulla and stimulates the release of catecholamines into the blood stream [48, 49]. Since, in respect of neurotransmitters, only urine is not a sufficient indicator of central nervous system (CNS) activity [50] hence, considering the potential role of plasma catecholamines as a readily available measure of sympathoadrenal system activity [51], the developed protocol has been implemented for the simultaneous determination of EP and NE in urine as well as plasma samples of athletes and smokers. Further, most of the research focuses on biochemical and medical aspect of catecholamines and comparatively few studies are available regarding their analytical aspect [52-54] hence, it is believed that simultaneous sensing of EP and NE in human body fluids will be of great importance to monitor nerve physiology, doping cases, controlling medicine in biomedical, biopharmaceutical research and clinical diagnosis of some aforementioned disorders [22, 55].

In comparison to other conventional electrodes EPPGE has been proved to be an effective substrate for detecting lower concentration of biomolecules and drugs due to its easy maintenance, low background current and wide potential window [56-58]. Square

wave voltammetry is a versatile technique for electroanalytical purposes as it has higher sensitivity and effectively suppresses background current. This chapter presents a novel approach using square wave voltammetry (SWV) with the combination of multi-walled carbon nanotubes modified edge plane pyrolytic graphite electrode as a sensitive and selective sensor for the determination of epinephrine and norepinephrine in human body fluids.



Epinephrine



Norepinephrine

3.2 EXPERIMENTAL

3.2.1 Instruments

Voltammetric experiments were performed using Bioanalytical System (BAS, West Lafayette, USA) CV-50W voltammetric analyzer coupled with a conventional three-electrode cell system. An edge plane pyrolytic graphite electrode or a MWNT modified edge plane pyrolytic graphite electrode served as working electrode. Ag/AgCl (3MNaCl) (BAS Model MF-2052 RB-5B) and platinum wire served as reference and auxiliary electrode, respectively. All potentials reported are with respect to Ag/AgCl at an ambient temperature of 25±2 °C. The edge plane pyrolytic graphite pieces were obtained from Pfizer Inc. New York, USA and the electrode was prepared as reported in literature [59]. The surface morphology of the bare and modified electrodes was characterized by recording FE-SEM using Quanta 200 FE-SEM (FEI Company) instrument. The pH of the buffer solutions was measured using Eutech Instruments pH 510, pH meter after standardization. HPLC studies were performed on Shimadzu LC-2010A HT system with RP-18e (5μm) column. The mobile phase composed of a mixture of acetonitrile: water (80:20) was delivered at a flow rate of 1.2 $mLmin^{-1}$ and detection was carried out at 210 nm.

3.2.2 Chemicals

Epinephrine, dopamine, norepinephrine, uric acid and ascorbic acid were purchased from Sigma Aldrich and used as received. The stock solutions of the compounds (1.0 mM) were prepared using double distilled water. The multi-walled carbon nanotubes (purity > 98%) was obtained from Bucky, USA. Adrenaline bi tartrate injection (G.K. Pharmaceuticals; Mfg. Lic. No. 5/SC/P-2006) was obtained from the Institute hospital of Indian Institute of Technology Roorkee. Other reagents used were of analytical grade. The urine and plasma samples of three high-nicotine cigarette smokers (male: 35 years, 66 kg, male: 50 years, 76 kg, male: 42 years, 55 kg) and nonsmokers as control (male: 30 years, 51 kg, male: 39 years, 64 kg, male: 40 years, 64 kg) in resting stage were collected after 15 min. of smoking from the hospital of Indian Institute of Technology, Roorkee after clearance from Ethics Committee of IIT Roorkee. The blood plasma samples of two athletes (Sample 1: female, age 24 yrs, height 152 cm, weight 52 kg; Sample 2: female, age 28 yrs, height 156 cm, weight 56 kg) were collected soon after peak exercise of ~ 30 min from the Institute hospital. Prior to recording voltammograms urine and blood plasma samples were diluted four and two times, respectively with phosphate buffer solution of pH 7.2 to minimize matrix complexity.

3.2.3 Analytical procedure

The stock solutions (1 mM) of epinephrine, norepinephrine, dopamine, uric acid and ascorbic acid were prepared by dissolving the required amount of the compound in double distilled water. Phosphate buffer solutions (1M) were prepared according to the method of Chiristian and Purdy [60]. Aliquots of the stock solutions of compounds were diluted with appropriate amount of phosphate buffer of desired pH for recording voltammograms. In order to deoxygenate the solutions for recording cyclic voltammograms, high-purity nitrogen was bubbled for 12–15 min. Cyclic voltammograms were recorded in the sweep range of 20–500 mVs^{-1} with initial sweep to positive potentials. The optimized parameters selected for square wave voltammetry were: initial E: −100 mV; final E: 1000 mV; square wave amplitude (E_{sw}): 25 mV; potential step (E): 4 mV; square wave frequency (*f*): 15 Hz.

3.2.4 Influence of amount of MWNT suspension

It was found that the amount of nanotubes casted on the electrode surface affects the efficient modification and voltammetric response of epinephrine. To determine the optimum amount of MWNT suspension different amount in the range 20 to 60 μL were casted at the

electrode surface. It was found that the peak current increase with increase in volume of MWNT casted upto 40 μL and then remained constant upto 60 μL. This behavior is related to the thickness of film. If the film was too thin then the amount of EP on the electrode surface was less, thus the response was comparatively poor. Therefore, 40 μL MWNTs suspension was chosen as the optimum amount for the surface modification of EPPGE.

3.2.5 Preparation of MWNT modified EPPGE

The surface of bare EPPGE was rubbed on an emery paper (P-600) prior to modification and then rinsed with double distilled water and dried. The multi-walled carbon nanotubes were dispersed in N, N-dimethylformamide (DMF) with the aid of ultrasonic agitation to prepare 0.5 mg/mL MWNTs suspension. Then modified electrode was fabricated by dropping 40 μL MWNT suspensions on bare EPPGE surface and then the solvent was allowed to evaporate at room temperature. The modified electrode surface was cleaned by applying a potential of − 200 mV for 60 s. The surface morphology of bare and MWNT modified EPPGEs is studied by recording FE-SEM images is presented in **Fig. 3.1 (A) and (B)**, respectively. Fig. 3.1 (B) clearly shows the deposition of MWNT at the surface of EPPGE.

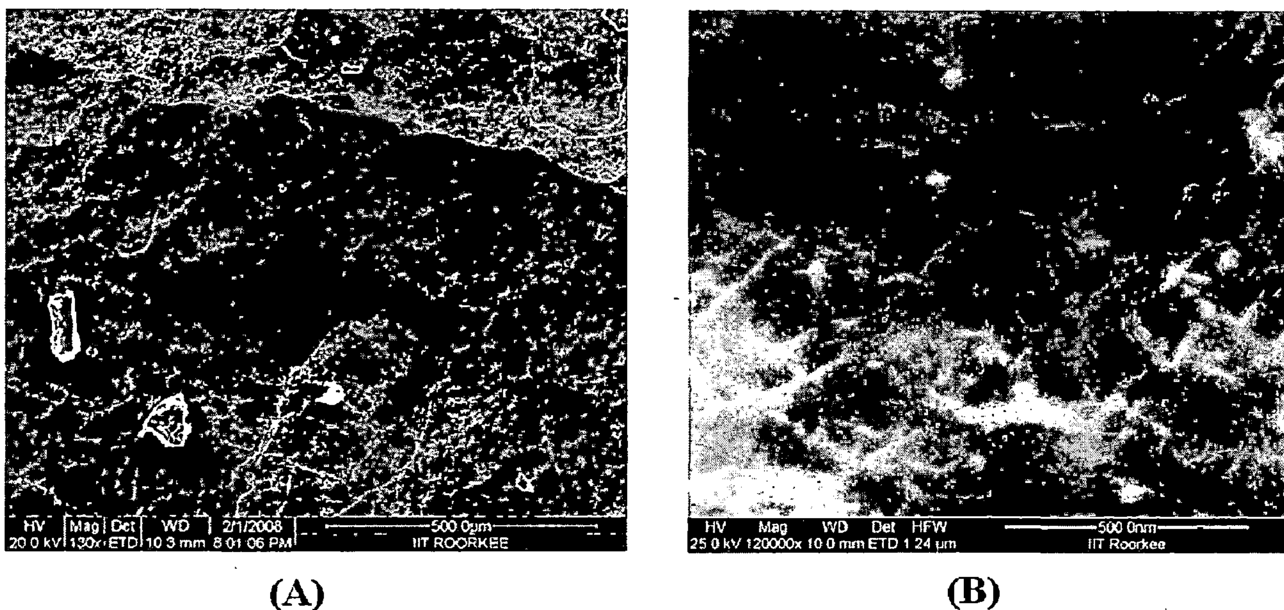


Fig. 3.1 FE SEM images of (A) bare and (B) MWNT modified edge plane pyrolytic graphite electrode.

3.2.6 Surface area

Higher surface area of an electrode is expected to impart higher electrocatalytic activity resulting higher sensitivity [61]. Cyclic voltammograms of 1 mM $K_3Fe(CN)_6$ containing 0.1 M KCl were recorded at different scan rates using bare EPPGE and MWNT/EPPGE in order to calculate surface area. A redox couple was observed due to the Fe^{+3}/ Fe^{+2} at both bare and modified electrodes and corresponding reversible processes follow the equation:

$$i_p = 0.4463\ (F^3/RT)^{1/2} A\ n^{3/2} D_R^{1/2} C_0\ \nu^{1/2}$$

where F is Faraday's constant (96485 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 (Ampere), n = 1 for $K_3Fe(CN)_6$, T is the absolute temperature (298 K), $D_R = 7.6 \times 10^{-6}$ $cm^2\ s^{-1}$, ν is scan rate (Vs^{-1}) and C_0 is the concentration of $K_3Fe(CN)_6$ in $molL^{-1}$. The surface area was calculated from the slopes of the i_p versus $\nu^{1/2}$ plots and found as 0.0529 and 0.1005 cm^2 for bare and MWNT modified EPPGE, respectively. Experimental results thus indicate that the surface area of MWNT modified EPPGE is almost 2-fold larger than the surface area of bare EPPGE.

3.3 RESULTS AND DISCUSSION

(A) DETERMINATION OF EPINEPHRINE

3.3.1 Electro-oxidation of epinephrine at bare EPPGE and MWNT/EPPGE

Square wave voltammograms of 60 nM epinephrine in phosphate buffer solution of pH 7.2 were recorded utilizing bare and modified electrodes. **Fig. 3.2** shows the SWVs of epinephrine using bare (curve a) and MWNT modified EPPGE (curve b) at pH 7.2. As can be seen, EP exhibits an oxidative peak ~198 mV at bare electrode whereas a comparatively well defined peak was observed ~150 mV and the peak current also increased at MWNT/EPPGE. Therefore, further detailed studies for EP analysis have been performed utilizing MWNT/EPPGE. The nanometer dimension, presence of metal impurities in nanotubes, electronic structure, and topological defects present on the nanotubes surfaces are some important reasons for the improved voltammetric response of MWNT modified EPPGE [62, 63].

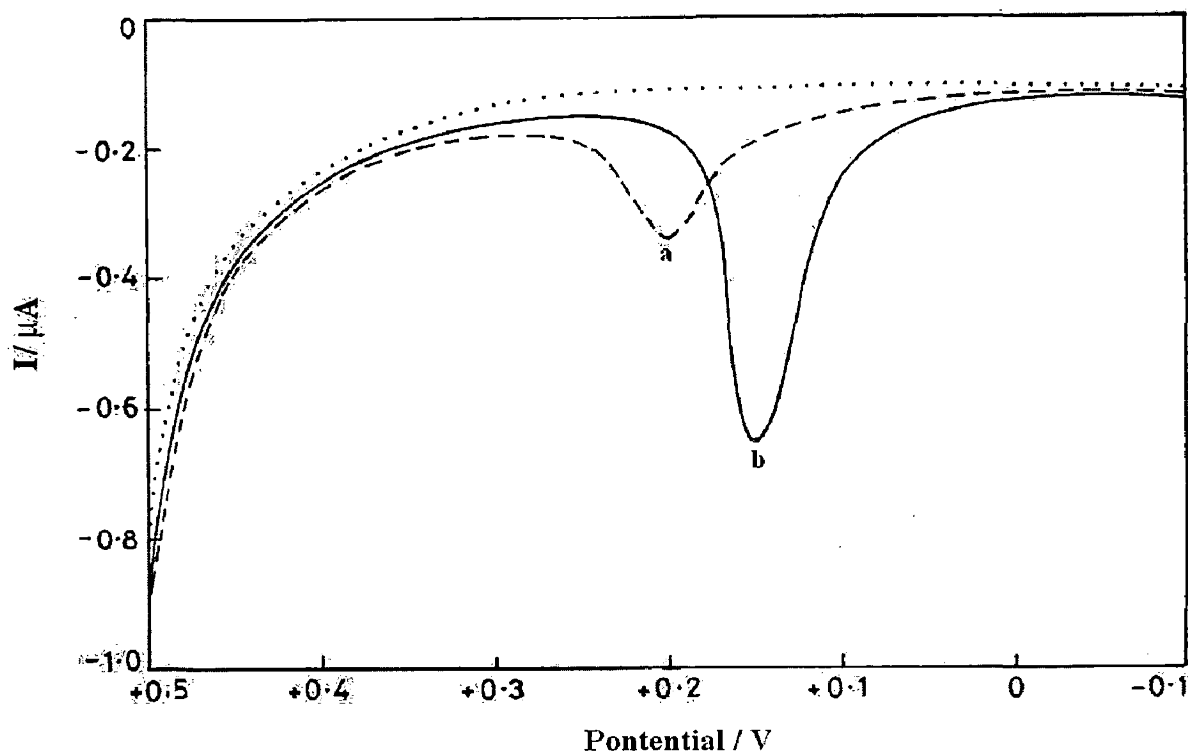


Fig. 3.2 Square wave voltammograms of 60 nM epinephrine at bare EPPGE (a) and MWNT modified EPPGE (b) in phosphate buffer solution of pH 7.20, and dotted line is the response of MWNT/EPPGE in PBS of pH 7.20 in the absence of EP.

3.3.2 Cyclic voltammetric behavior of epinephrine

The electrooxidation is closely related to the solution pH hence, cyclic voltammograms of EP were recorded at different pH values using MWNT/EPPGE. **Fig. 3.3** presents the electrochemical behavior of 5 μM epinephrine at pH 7.20 using MWNT/EPPGE at 20 mVs^{-1}. The cyclic voltammogram exhibits an irreversible peak at 165 mV (I_a) due to the oxidation of epinephrine to o-quinone and a reversible couple with peak potentials of – 216 (II_c) and – 194 (II_a) mV which is attributed to the formation of epinephrinechrome/leucoepinephrinechrome redox couple [64]. In acidic media (pH 2.37), the peak I_a exhibited a quasi reversible peak I_c which is attributed to the reduction of epinephrinequinone to epinephrine however, the reverse couple II_a / II_c was not observed as shown in **Fig. 3.3** Thus, it is interesting to observe that with increase in pH, the tendency of formation of epinephrine / quinone redox couple decreases. Also in neutral pH range the

epinephrinequinone; product of epinephrine oxidation reaction go to cyclization through deprotonation and convert to adrenochrome via a series of chemical reactions [36]. In acidic media (pH 2.37) deprotonation is not favorable hence; reversible reaction become favorable and epinephrinequinone get reduced to epinephrine corresponding to redox couple of peaks (Ia) and (Ic). However, at pH 7.20, epinephrine get irreversibly oxidized to epinephrinequinone corresponding to peak Ia, since at pH 7.20 it is comparatively easy to lose proton through cyclization which gives adrenochrome. The reduction peak at – 216 (II_c) at pH 7.2 is attributed to the reduction of the cyclized product adrenochrome to leucoadrenochrome while, peak at – 194 (II_a) is that of the re-oxidation of leucoadrenochrome to adrenochrome.

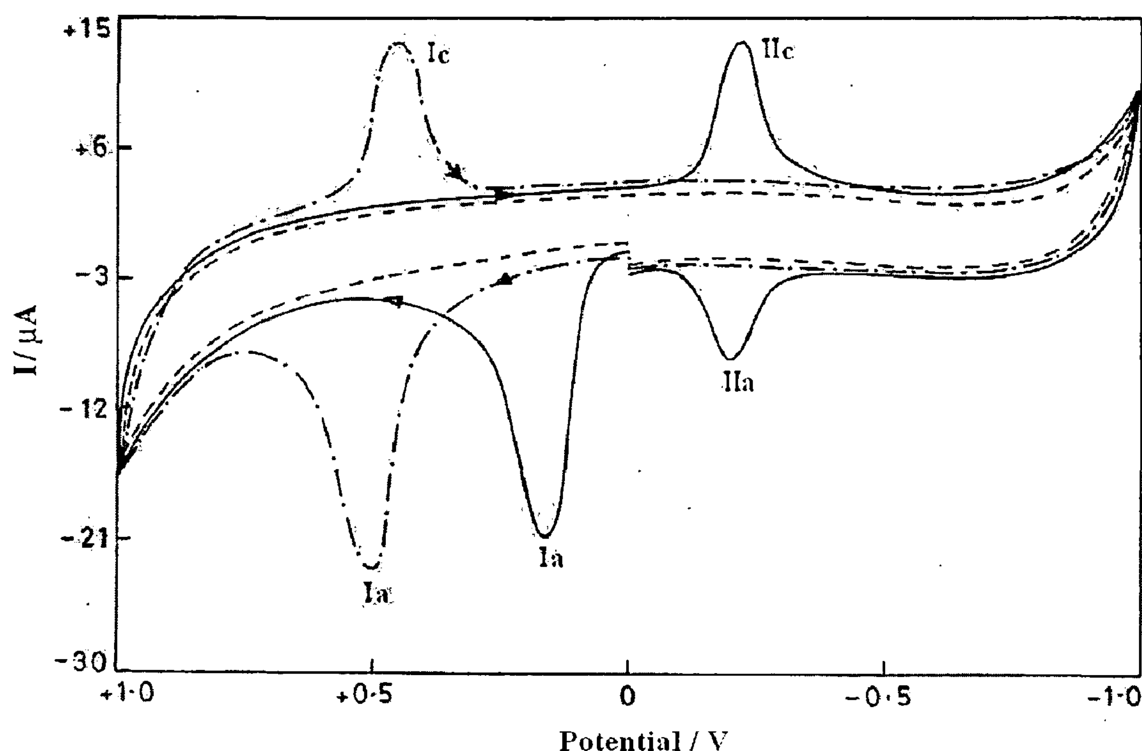


Fig. 3.3 Cyclic voltammograms of (a) 5 μM epinephrine solution at pH 7.20 (——) and (b) 10 μM epinephrine solution at pH 2.37 (—•—•) at scan rate of 20 mVs^{-1} and dashed CV (- - - -) is the response of MWNT/EPPGE in blank PBS.

The effect of pH on peak I_a, corresponding to epinephrine to epinephrinequinone oxidation, at scan rate 20 mVs^{-1} was also studied. It was found that the peak potential for

peak I_a shifted to more negative potentials with increasing pH. The variation of peak potential with pH was linear and obeyed the following equation:

$$E_p\ (mV) = -64.77\ pH + 650.2$$

having a correlation coefficient of 0.996. The dEp/dpH value ~ 65 mV/pH indicates that the electrons transfer was accompanied by an equal number of protons in the oxidation reaction of epinephrine to epinephrinequinone [65].

The influence of scan rate on epinephrine (1 μM) oxidation peak Ia was studied by recording cyclic voltammograms in the range of 20 – 500 mVs^{-1} utilizing MWNT/EPPGE at pH 7.20. It was found that the peak current increases linearly with the square root of scan rate and dependence of peak current on scan rate can be expressed by the equation:

$$i_p\ (\mu A) = 0.374\ \nu^{1/2} + 2.803$$

where ν is scan rate in mVs^{-1} having correlation coefficient 0.997. These results indicate that the electron transfer reaction is controlled by the diffusion of epinephrine at MWNT/EPPGE, which is favorable for quantitative determination. The graph between log i_p *vs.* log ν was also found to be linear having slope value < 0.5 which further confirm the diffusion controlled reaction [66].

3.3.3 Calibration plot

Square wave voltammograms were recorded using MWNT/EPPGE for different concentration of epinephrine in order to plot calibration curve at pH 7.20. A well-defined peak was observed at ~150 mV during oxidation of epinephrine using MWNT/EPPGE. The variation of peak current with epinephrine concentration was investigated under the optimum experimental parameters in order to plot calibration curve. The peak current is found to increase linearly with increasing concentration of epinephrine from 0.5×10^{-9} to 0.1×10^{-6} M and the linear relation is expressed by the regression equation:

$$i_p\ (\mu A) = 8.699\ C + 0.0198$$

where C is concentration (μM) having correlation coefficient 0.998. The limit of detection and sensitivity were found to be 0.15×10^{-9} M (S/N=3) and 8.7 μA/μM, respectively. These results suggest that the electrode is quite sensitive to epinephrine determination although it can be easily prepared.

3.3.4 Interference study

Epinephrine often exists in natural environments together with high concentration of electroactive biomolecules like uric acid that interfere with each other [67]. Hence, in order to examine the selectivity of MWNT/EPPGE for epinephrine determination the effect of some common interferents such as uric acid, ascorbic acid, dopamine and norepinephrine was evaluated. For this purpose square wave voltammograms of a solution having different concentrations of standard ascorbic acid (AA), dopamine (DP), epinephrine (EP), norepinephrine (NE) and uric acid (UA) were recorded at pH 7.20. Five well separated peaks at ~ − 50, 80, 150, 204 and 260 mV were observed corresponding to the oxidation of ascorbic acid, dopamine, epinephrine, norepinephrine and uric acid, respectively. As compared to catecholamines, comparatively high concentrations of uric acid and ascorbic acid were choosed for interference study owing to the fact that these are major biomolecules of body fluids. In order to further confirm the selectivity of the modified electrode for epinephrine determination the concentrations of epinephrine was increased in a synthetic mixture having constant amount of uric acid, ascorbic acid, dopamine and norepinephrine as shown in **Fig. 3.4**. Moreover, the selectivity of modified sensor was over again examined by increasing the concentration of each interfering substance from 5 to 1000 fold when epinephrine concentration was constant. The experiment results show that no substantial changes in peak current response of epinephrine was observed for entire concentration range of uric acid, ascorbic acid and dopamine. However, norepinephrine interferes severely when its concentration was more than 100 fold of epinephrine. Since, in body fluids the concentration of norepinephrine is nearly comparable to epinephrine hence no matter if it interferes tills 100 times of epinephrine. Therefore, it is concluded that MWNT/EPPGE can be safely used for epinephrine analysis in biological samples as well as in pharmaceutical preparations.

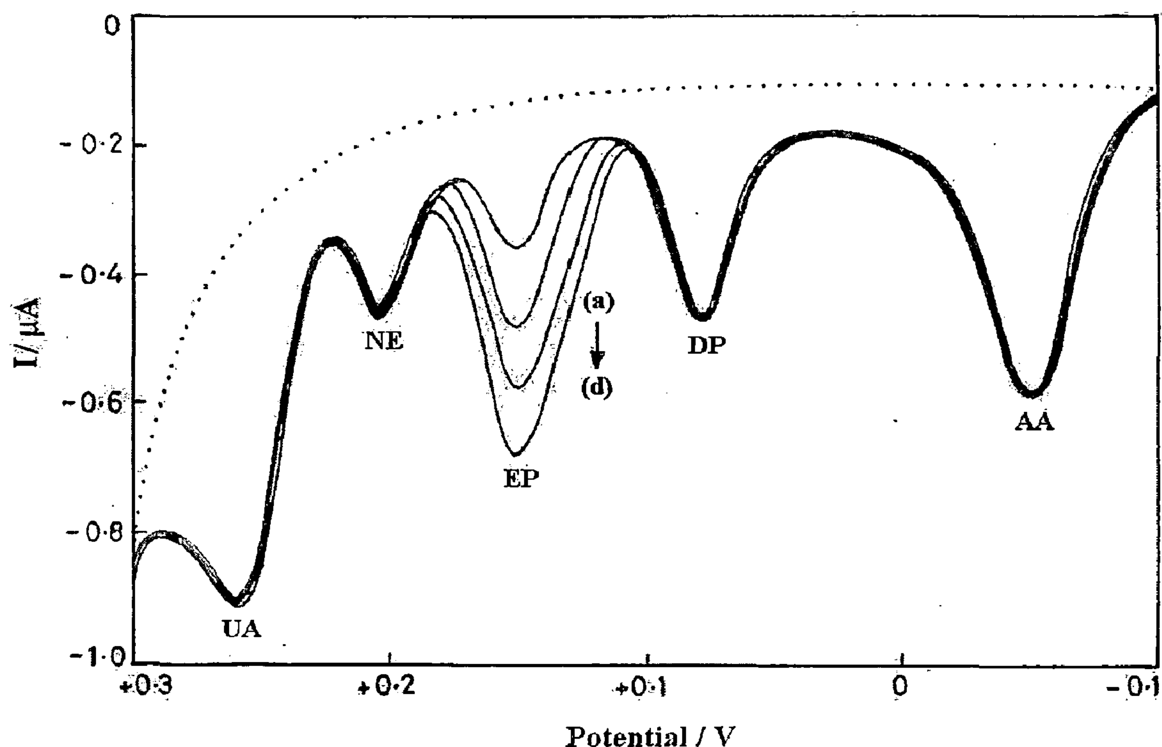


Fig. 3.4 Square wave voltammograms of synthetic mixture having constant concentration of ascorbic acid, dopamine, norepinephrine and uric acid, and increasing concentration of epinephrine [(a) = 25, (b) = 40, (c) = 50, (d) = 60 nM] in PBS of pH 7.20 using MWNT/EPPGE. The dotted line is the response of MWNT/EPPGE in blank PBS of pH 7.20.

3.3.5 Analytical utility

3.3.5.1 Biological sample analysis

A close relation between the release of epinephrine and smoking is reported in literature [48], therefore, the proposed sensor has been utilized for the analysis of epinephrine in body fluids of smokers and nonsmokers. Owing to the fact that analysis of only urine sample is not a sufficient indicator of CNS activity and plasma sample also should be analyzed especially in case of neurotransmitters [50]; plasma samples of three healthy smokers and nonsmokers in resting stage were analyzed utilizing MWNT/EPPGE. Prior to analysis samples were diluted 2 times with phosphate buffer solution of pH 7.2 in order to reduce matrix effect and then SWVs were recorded. It was found that no peak was observed for plasma samples of control subjects in specific potential region of catecholamines whereas, a small peak (E_p ~150 mV) appeared together with two other small

peaks at ~ 80 and ~ 204 mV in plasma samples of smokers. The fact that two other peaks at ~ 80 and ~ 204 mV are due to the oxidation of dopamine and norepinephrine, respectively was confirmed by recording the voltammograms of their authentic samples. The plasma sample of smoker was then spiked with a known concentrations of epinephrine and it was found that the peak current increases significantly for the peak at E_p ~150 mV thereby, confirming that the peak at 150 mV corresponds to the oxidation of epinephrine as shown in **Fig. 3.5**. The actual concentration of epinephrine in plasma samples 1 of smoker was calculated by using regression equation and considering dilution factor it was found to be 0.50×10^{-9} M. Square wave voltammograms recorded for plasma sample 2 and 3 of smokers also depict similar results. These three plasma samples of smokers were then spiked with 0.5 and 1.0 nM concentration of standard epinephrine to reconfirm the actual concentration present in smokers' plasma samples. Further, HPLC study was performed to determine epinephrine concentration in plasma samples of three smokers in order to prove the reliability of data obtained by proposed method.

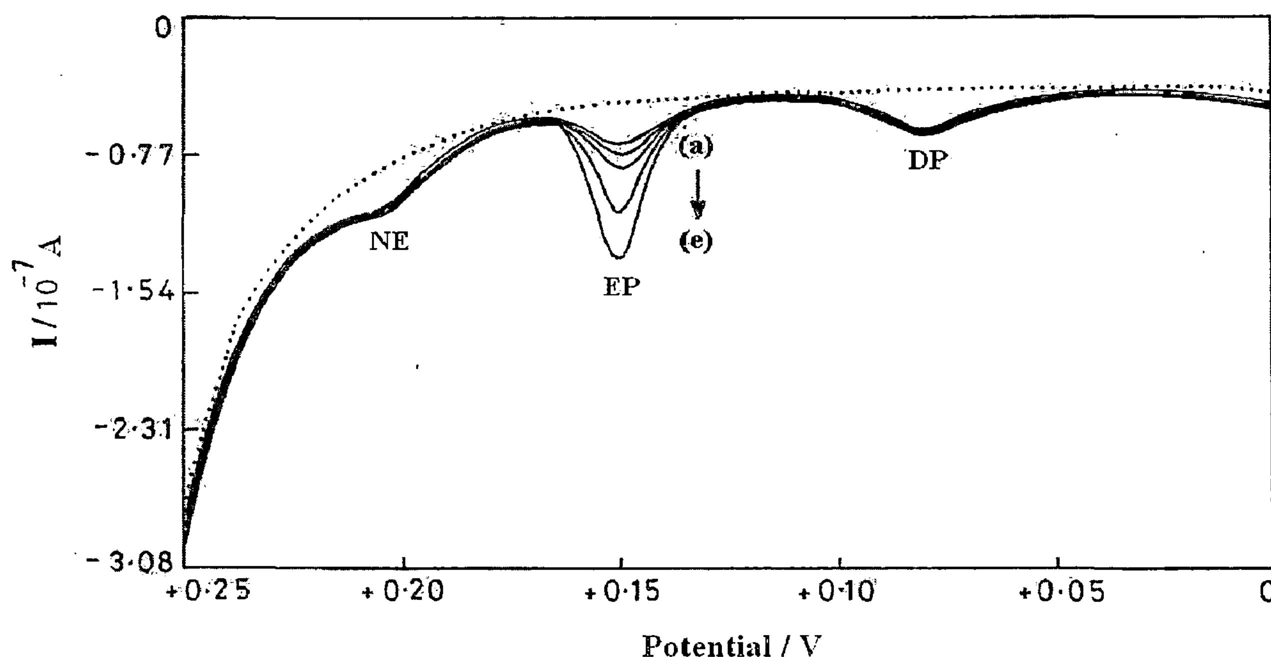


Fig. 3.5 Square wave voltammograms of (i) blank PBS (.....) and (ii) plasma sample 1 of smoker (a), plasma sample 1 of smoker spiked with 0.50 (b), 1.50 (c), 4.50 (d), 7.50 (e) nM epinephrine at pH 7.2 using MWNT/EPPGE.

Table 3.1 shows the results obtained for epinephrine determination in plasma samples of smokers by using proposed sensor and HPLC. The results show that recovery data of the spiked samples are in acceptable range and results are also comparable with well known HPLC method. The developed method has also much lower detection limit (0.15×10^{-9} M) and high sensitivity (8.699 μA/μM) alongwith significant practical utility as compared to the recently reported methods **(Table 3.2)**. Hence, it is concluded that the newly proposed method for determination of epinephrine has good accuracy and promising applications.

Table 3.1 Square wave voltammetric determination of epinephrine in plasma samples of healthy smokers at MWNT/EPPGE by SWV and by using HPLC

Spiked amount (nM)	Detected amount (nM)*	Actual amount (nM)		Recovery (%)
		MWNT/EPPGE	HPLC	
Sample 1				
0.00	0.25	0.25	0.28	—
0.50	0.76	0.26	0.22	101.33
1.00	1.74	0.24	0.26	99.43
Sample 2				
0.00	0.28	0.28	0.30	—
0.50	0.77	0.27	0.24	98.72
1.00	1.80	0.30	0.34	101.12
Sample 3				
0.00	0.22	0.22	0.20	—
0.50	0.74	0.24	0.21	102.78
1.00	1.70	0.20	0.26	98.84

* The R.S.D. value for the determination of epinephrine was less than ± 2.8 % for n=3

Table 3.2 A comparison of voltammetric response of MWNT/EPPGE with previously reported electrodes for the determination of epinephrine

Electrode	Concentration range (μM)	Detection limit (μM)	Sensitivity (μA/μM)	Real samples Plasma	Real samples Urine	Ref. no.
50% (m/m) SWNT-CPE	0.40 – 55.0	0.20	0.022	No	No	[29]
LDHf/GCE	0.50 – 300	1.00	0.737	No	No	[30]
Dopamine/Au electrode	2.0 – 800	0.30	0.123	No	No	[31]
Au-Cys-SWNT-CoTAPc	12.2 – 130	6.00	0.0094	No	No	[32]
Paraffin/MWNT/CoPC	1.33 – 5.50	0.015	6.29	No	Yes	[33]
HMWCNT/GCE	0.20 – 78.3	0.024	0.065	No	Yes	[34]
Sonogel-L-Cys. Elec.	0.10 – 500	0.087	0.0521	No	Yes	[35]
Al-MCM-41/CPE	0.8 – 100	0.03	—	No	Yes	[36]
GC/Ni/PU-C Elec.	0.10 – 10.0	0.01	0.90	No	Yes	[37]
2 PHCMCNPE	0.05 – 0.95	0.0096	7.749	No	No	[38]
NDG/PG	0.01–10.0	0.003	7.095	No	Yes	[39]
MWNT/BPPGE	0.10 – 100	0.02	0.42	No	No	[40]
MWNT/EPPGE	0.0005 – 0.100	0.00015	8.699	Yes	Yes	Proposed method

3.3.5.2 Pharmaceuticals analysis

The analytical utility of proposed sensor was further examined by analysis of adrenaline bi tartrate injections. The content of the injection sample of different batches was suitably diluted with 1M phosphate buffer solution of pH 7.20 before the measurements so that reported epinephrine concentration falls in the range of calibration plot. The concentration of epinephrine in injections sample determined by employing the proposed method is compared with labeled concentration of epinephrine and is found in agreement with that reported on the label of the pharmaceutical product. This determination procedure

was repeated four times and the relative standard deviation was 2.6% hence, indicated the adequate accuracy of the proposed method. In order to reconfirm the accuracy of the proposed method it was considered worthwhile to determine epinephrine concentration in adrenaline bi tartrate injection sample by HPLC method. It was found that results of proposed method are not only comparable with labeled value on the pharmaceutical product but also with HPLC results.

(B) SIMULTANEOUS DETERMINATION OF EPINEPHRINE AND NOREPINEPHRINE

3.3.6 Cyclic voltammetry

Initially, cyclic voltammograms were recorded for 5 μM epinephrine or norepinephrine after bubbling high-purity nitrogen for 12-15 min. using MWNT/EPPGE at pH 7.2. The cyclic voltammogram of EP exhibits an irreversible peak at 170 mV (I_a) when the sweep was initiated in positive direction. In the reverse sweep, a reduction peak (II_c) was noticed which formed a reversible couple with peak II_a, observed in second positive sweep. The peak potentials of the couple were – 216 (II_c)/– 194 (II_a) mV **(Fig. 3.6)**. Cyclic voltammogram of NE also exhibited an oxidation peak at 220 mV (I_a) in the initial positive sweep. In the reverse sweep it exhibits two reduction peaks at 183 (I_c) and -182 (II_c) mV. Peak I_c formed a quasi reversible couple with peak I_a. On further reversing the direction a peak at -154 mV (II_a) was observed, which formed a redox couple with peak (II_c) **(Fig. 3.6)**. Finally, a cyclic voltammogram of binary mixture of 5 μM each EP and NE was recorded and oxidation peaks as well as reduction peaks of EP were found to merge with NE (Fig. 2). Thus, simple voltammetry was unsuccessful to resolve the two peaks of NE and EP. As square wave voltammetry is more sensitive technique in comparison to cyclic voltammetry, hence it is used to resolve overlapped voltammetric response of epinephrine and norepinephrine.

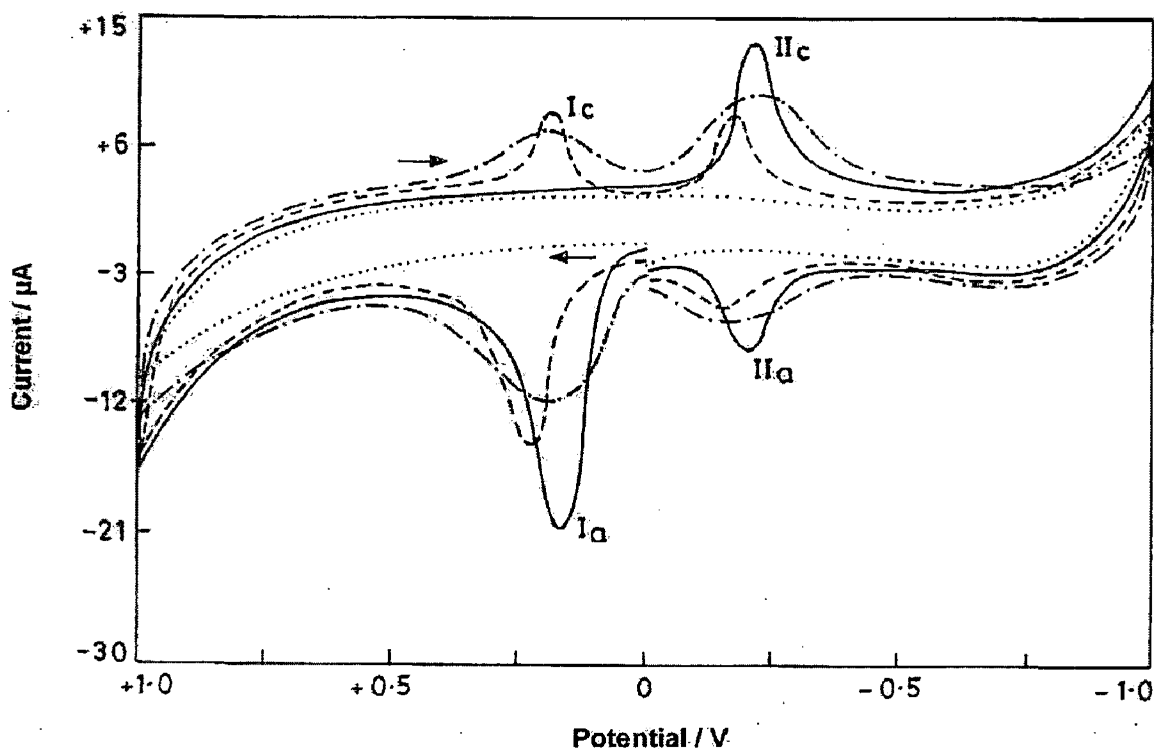


Fig. 3.6 Cyclic voltammograms of 5 μM epinephrine solution (——), 5 μM norepinephrine solution (- - - -), mixture of 5 μM EP and NE (–·–·–·) and blank PBS of pH 7.2 (……) using MWNT/EPPGE at scan rate of 20 mVs^{-1}.

3.3.7 Comparison of bare EPPGE and MWNT/EPPGE

Initially square wave voltammograms were recorded for a binary mixture of epinephrine and norepinephrine at bare and modified EPPGE working electrodes in phosphate buffer solution of pH 7.2 as shown in **Fig. 3.7**. At the bare edge plane pyrolytic graphite electrode, the voltammetric response is poor with a large voltammetric bump at ~250 mV. While the much improved voltammetric response having two well separated voltammetric peaks at ~150 and ~215 mV for epinephrine and norepinephrine, respectively was obtained at MWNT/EPPGE. Thus, **Fig. 3.7** indicates that MWNT/EPPGE serves as a better substrate for the simultaneous determination of EP and NE neurotransmitters. The two well-separated peaks with shift of the peak potential towards less positive potential in conjunction with a significant increase in peak current at MWNT modified EPPGE revealed that the proposed voltammetric sensor acts as a very efficient promoter to enhance the

kinetics of the electrochemical process. Hence, MWNT/EPPGE has been utilized for further detailed studies of catecholamines.

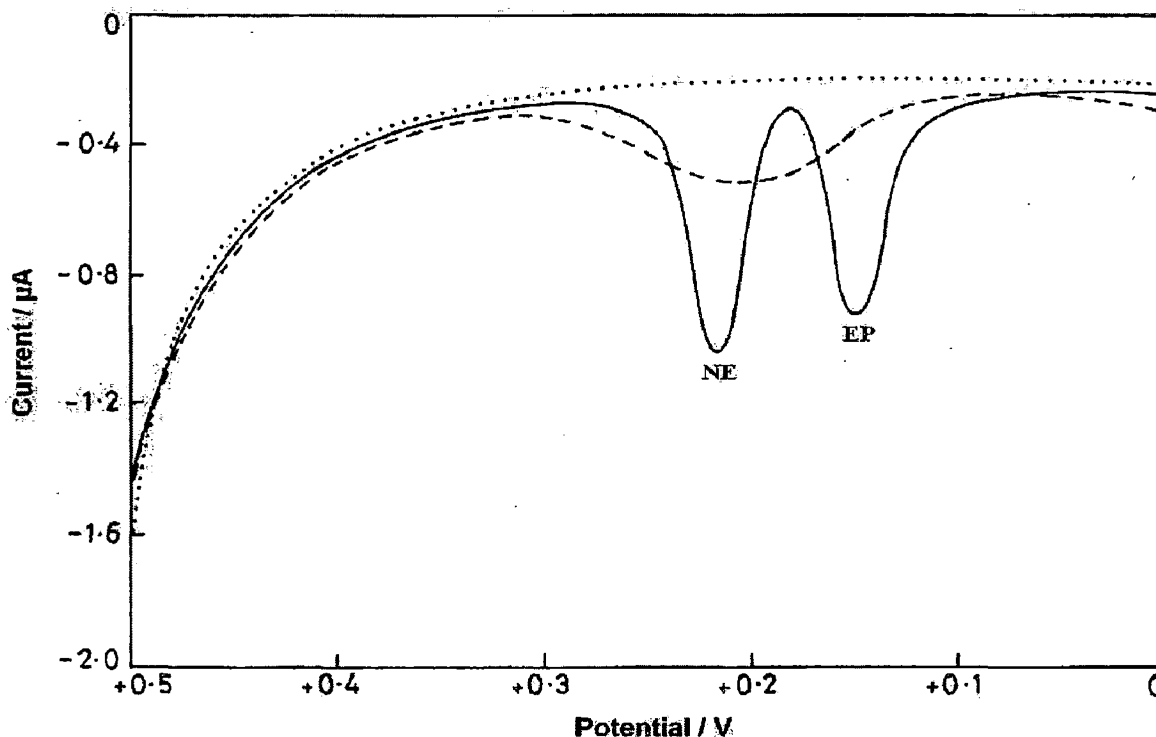


Fig. 3.7 Square wave voltammograms of binary mixture of epinephrine (80 nM) and norepinephrine (60 nM) using bare EPPGE (- - - -) and MWNT modified EPPGE (—) at pH 7.20 and dotted line is the response of MWNT/EPPGE in blank PBS of pH 7.20.

3.3.8 Electrochemical behavior of epinephrine and norepinephrine

3.3.8.1 Effect of pH

The effect of pH on the oxidation of EP was studied in the pH range 2.4–8.8 using square wave voltammetry. It was found that the peak potential shifted towards less positive potentials with increase in pH and the variation of peak potential (E_p) with pH was linear. The dependence of E_p on pH obeys the relation:

$$E_p \text{ (mV vs. Ag/AgCl)} = 616.7 - 63.46 \text{ pH}$$

having correlation coefficient 0.998. Similarly the oxidation potential of NE was also found to shift towards less positive potentials with increase in pH. The linear dependence of the peak potential on pH is represented by the following equation:

$$E_p \text{ (mV vs. Ag/AgCl)} = 669.6 - 62.26 \text{ pH}$$

having correlation coefficient of 0.998. The slope of E_p versus pH plots for EP and NE is close to 60 mV/pH and hence suggests that equal number of protons and electron are involved in the electrode reaction [65].

3.3.8.2 Effect of square wave frequency

The dependence of oxidation peak current (i_p) on the square wave frequency (f) for EP or NE was studied in the range of 5–100 Hz. The peak current was found to increase linearly with square wave frequency (f) (Hz) for both the analytes. The linear relation between i_p and $f^{1/2}$ for EP can be expressed by the equation:

$$i_p\ (\mu A) = 0.255\ (f)^{1/2} - 0.471$$

having correlation coefficient 0.998. On the same way the relation between i_p and $f^{1/2}$ for NE can be represented by the following equation:

$$i_p\ (\mu A) = 0.406\ (f)^{1/2} - 0.693$$

having correlation coefficient 0.997. These observations indicate that the reactions occurred at the surface of MWNT/EPPGE are governed by the diffusion controlled process for both the catecholamines [68].

3.3.8.3 Concentration study

Square wave voltammograms for different concentrations of EP were recorded in phosphate buffer solution of pH 7.2 and the peak current increased with increase in concentration of EP. The peak current (i_p) was found to be linearly dependent on concentration in the range of 0.5–100 nM **{inset Fig. 3.8 (A)}**. The linear regression equation having correlation coefficient 0.998 is presented as:

$$i_p\ (\mu A) = 0.0087\ C\ (\text{nM}) + 0.0198$$

where C is the concentration of EP. The detection limit was calculated by using the relation $3\sigma/b$, where σ is the standard deviation of the blank and b is the slope of the calibration curve. The detection limit and sensitivity of EP determination was calculated to be 0.15

$\times 10^{-9}$ M (S/N=3) and 8.7 μA/μM, respectively. The limit of quantification was found to be 0.48×10^{-9} M. The peak current of NE was also found to increase linearly with increasing concentration **{inset Fig. 3.8 (B)}** and the linear regression equation is expressed as:

$$i_p\ (\mu A) = 0.013\ C\ (nM) + 0.024$$

where C is the concentration of NE. The correlation coefficient for the expression was 0.996. The detection limit and sensitivity of NE determination was calculated to be 0.90×10^{-10} M (S/N=3) and 13.0 μA/μM, respectively and the limit of quantification for NE determination was calculated to be 0.28×10^{-9} M.

3.3.9 Simultaneous determination of epinephrine and norepinephrine

The main objective of the present investigation is to simultaneously determine the concentration of EP and NE. The MWNT modified EPPGE was utilized for this purpose and in the first set of experiments, concentration of EP was kept constant at 10 nM and NE was varied in the concentration range 0.5–100 nM as shown in **Fig. 3.8 (A)**. It can be seen that the oxidation peak of epinephrine is unaltered by the addition of NE and the peak height of NE increased with increase in its concentration. Similarly, while varying the concentration of EP in the concentration range 0.5–120 nM and keeping the concentration of NE fixed at 5 nM, the oxidation peak current of EP increases as depicted in **Fig. 3.8 (B)**. The current observed in both the cases for varied components were same as observed during the individual compound study and obeyed the calibration plot. Thus, the proposed method can be successfully used for the simultaneous determination of epinephrine and norepinephrine. These interesting and new results promoted us to use the proposed voltammetric sensor for the simultaneous determination of epinephrine and norepinephrine in human body fluids which is still aforementioned and also of great importance to monitor nerve physiology, doping cases, controlling medicine in biomedical and biopharmaceutical research [22, 55].

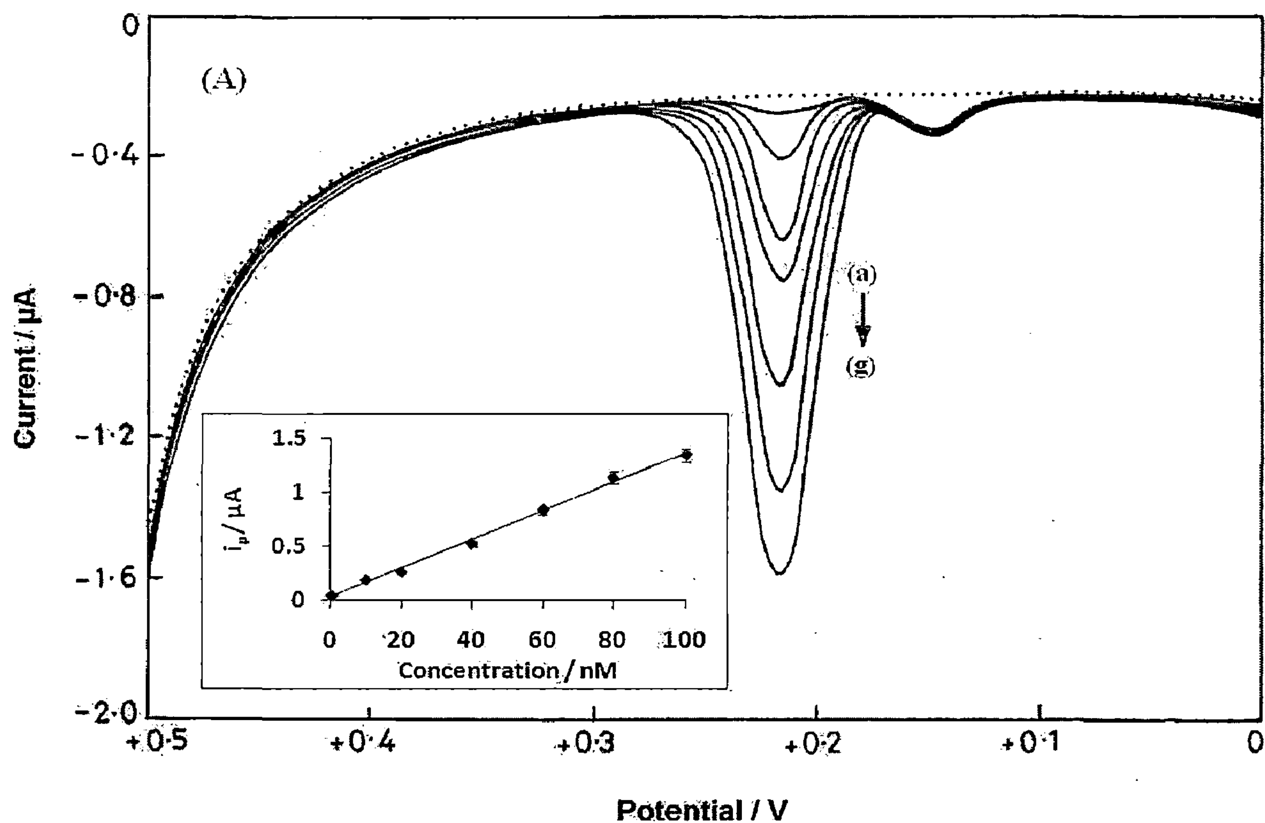


Fig. 3.8 (A) Square wave voltammograms of a binary mixture of EP and NE, keeping the concentration of EP constant (10 nM) and concentration of NE was (a) 0.5, (b) 10, (c) 30, (d) 40, (e) 60, (f) 80 and (g) 100 nM at pH 7.2. Inset is calibration curve for norepinephrine.

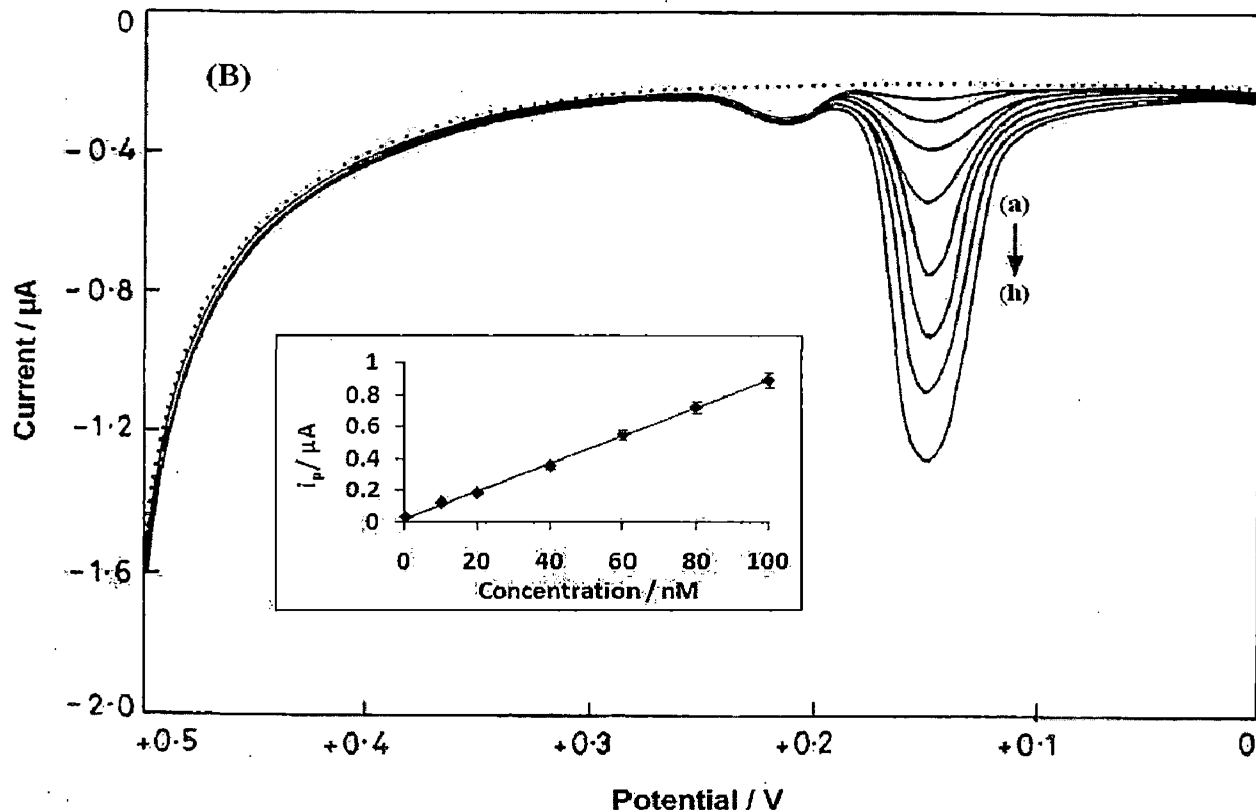


Fig. 3.8 (B) Square wave voltammograms of a binary mixture of EP and NE, keeping the concentration of NE constant (5 nM) and concentration of EP was (a) 0.5, (b) 5, (c) 20, (d) 40, (e) 60, (f) 80, (g) 100 and (h) 120 nM at pH 7.2. Inset is calibration curve for epinephrine.

3.3.10 Analytical utility of proposed sensor

The release of catecholamines in human system depends on smoking and exercise [48, 49]. Therefore, the proposed sensor has been examined for the simultaneous determination of EP and NE in body fluids of athletes and smokers.

3.3.10.1 Simultaneous determination of epinephrine and norepinephrine in human urine

The square wave voltammograms of urine samples of three smokers and nonsmokers (control) in resting stage were recorded under optimized parameters. To overcome the interference of major urinary metabolite viz. uric acid and ascorbic acid, an optimized potential region 0 to 250 mV was selected for the determination of catecholamines. The authentic sample of uric acid oxidized at ~300 mV, whereas, ascorbic acid was found to oxidize at ~ − 50 mV using MWNT/EPPGE. Square wave

voltammogram of urine sample 1 of nonsmoker healthy volunteer in resting stage (used as control) clearly shows three well separated peaks at ~80, ~150 and ~214 mV as shown in (solid line curve) **Fig. 3.9**. Two well-separated peaks at ~150 and ~214 mV were appeared corresponding to the oxidation of epinephrine and norepinephrine, respectively was confirmed by the standard addition method. Third peak at ~80 mV was found due to the oxidation of dopamine (DP) which was also confirmed by standard addition method although; no attempts have been made to determine its actual concentration. Further, square wave voltammograms of urine samples of smokers were recorded and three peaks were observed exactly at the same potentials as that observed in the case of control urine. However, the peak height of all the three peaks increased significantly as shown in (dashed line curve) **Fig. 3.9**. The actual concentrations of EP and NE were detected by using regression equation and in control subjects were found to be 65 $\times$ 10^{-9} and 12 $\times$ 10^{-8} M respectively, whereas, the concentrations of EP and NE in urine samples of smokers were found to be 105 $\times$ 10^{-9} and 18 $\times$ 10^{-8} M, respectively.

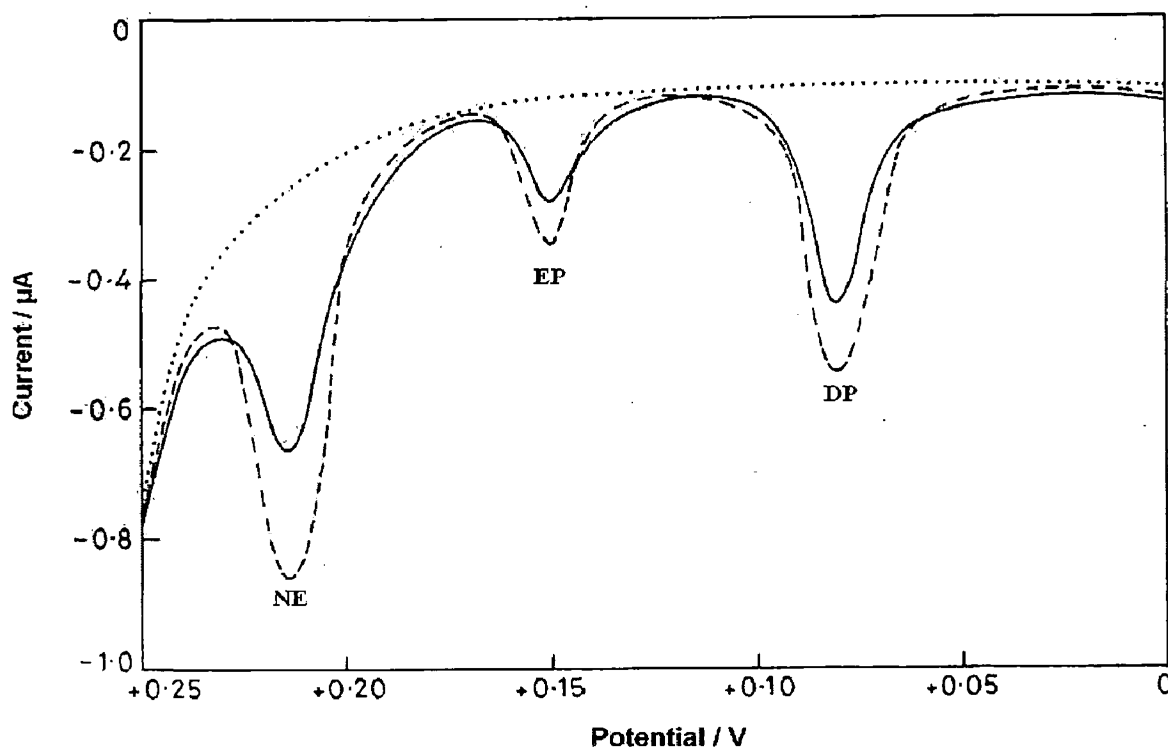


Fig. 3.9 Square wave voltammograms of (i) phosphate buffer solution (.....) and (ii) urine sample of healthy nonsmoker as control (—) and (iii) urine sample of healthy smoker (- - - -) at pH 7.2 using MWNT/EPPGE.

3.3.10.2 Simultaneous determination of epinephrine and norepinephrine in blood plasma

In the case of neurotransmitters, analysis of only urine sample has been considered not a sufficient indicator of CNS activity [50]. Therefore, considering the potential role of plasma catecholamines as a readily available measure of sympathoadrenal system activity the analysis of plasma samples is recommended [51]. Hence, the developed protocol has also been implemented for the determination of EP and NE level in blood plasma samples. For this purpose, square wave voltammograms of plasma samples of two smokers in resting stage and two athletes at peak exercise stage were recorded. These samples were spiked with known concentrations of standard epinephrine and norepinephrine and then voltammograms were recorded. The actual concentrations of EP and NE in plasma samples of smokers and athletes were determined by using standard addition method and results obtained are tabulated in **Table 3.3**. The results show that recovery data are in the range of 97.14 to 104.08 % and relative standard deviation (RSD) is less than ± 3.2 % hence, it can be concluded that proposed voltammetric sensor can be safely used for the simultaneous determination of EP and NE in human body fluids with excellent selectivity and sensitivity.

Table 3.3 Simultaneous determination of epinephrine and norepinephrine in plasma samples of smokers and athletes using MWNT/EPPGE

	Epinephrine (nM)			Norepinephrine (nM)		
Added	**Found***	**Actual**	**Recovery**	**Found***	**Actual**	**Recovery**
Smoker's Sample 1						
0.00	0.25	0.25	—	0.90	0.90	—
0.50	0.76	0.26	101.33	1.36	0.86	97.14
1.00	1.74	0.24	99.43	2.38	0.88	99.16
Smoker's Sample 2						
0.00	0.22	0.22	—	0.85	0.85	—
0.50	0.74	0.24	102.78	1.39	0.89	102.96
1.00	1.70	0.20	98.84	2.40	0.90	102.13
Athlete's Sample 1						
0.00	1.50	1.50	—	4.50	4.50	—
0.50	1.98	1.48	99.00	4.95	4.45	99.00
1.00	3.02	1.52	100.67	5.98	4.48	99.67
Athlete's Sample 2						
0.00	1.48	1.48	—	4.40	4.40	—
0.50	2.02	1.52	102.02	5.10	4.60	104.08
1.00	3.00	1.50	100.67	6.00	4.50	101.69

* The R.S.D. value for the determination of EP and NE was less than ± 3.2 % for n=3.

3.3.11 Validation of method

The results obtained by proposed voltammetric method were compared with independent HPLC method's results to prove the reliability of data obtained. For this purpose, HPLC chromatograms were recorded for different known concentrations of epinephrine and norepinephrine and observed peak area was plotted against their concentration. Linear calibration curves were obtained by plotting the peak area of analytes peaks against the analytes concentration. Two well defined peaks were obtained at Rt ~4.542 and ~5.729 min. in the standard sample of norepinephrine and epinephrine,

respectively. Then urine sample 1 of nonsmoker used as control was injected in HPLC instrument and it was found that norepinephrine and epinephrine peaks appeared at Rt ~ 4.542 and ~5.729 min. alongwith some other prominent peaks as indicated in **Fig. 3.10 (a)**. The additional peaks are likely to be due to uric acid, dopamine etc. although, no efforts have been made to characterize them. Urine sample 1 of smoker was then injected in HPLC instrument and peak area was found to be increased for epinephrine peak at Rt ~ 5.729 min and norepinephrine peak at Rt ~ 4.542 as shown in **Fig. 3.10 (b)**. Three urine samples of smokers and nonsmokers were analyzed to reconfirm the results obtained. Epinephrine and norepinephrine concentration in urine samples of three smokers and nonsmokers was determined by using calibration curve for HPLC study and results are tabulated in **Table 3.4**. A comparison of the values obtained by HPLC and the proposed voltammetric method clearly indicated that the results obtained by two methods are in good agreement.

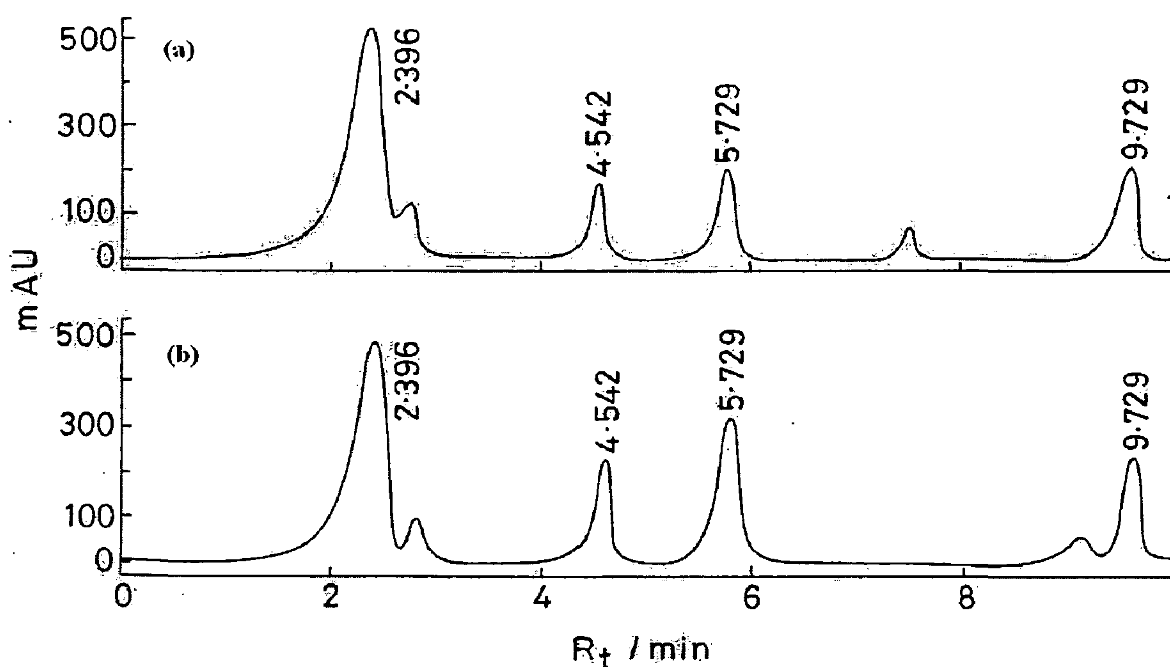


Fig. 3.10 Typical HPLC chromatograms observed for (a) urine sample of healthy nonsmoker as control and (b) urine sample of healthy smoker.

Table 3.4 Comparison of observed concentration of EP and NE in urine samples of nonsmokers and smokers at MWNT/EPPGE by SWV and by using HPLC

	Epinephrine (nM)		Norepinephrine (nM)	
Samples	**SWV**	**HPLC**	**SWV**	**HPLC**
Nonsmoker				
1	65.0	68.0	120.0	125.0
2	69.0	67.0	124.0	120.0
3	62.0	64.0	118.0	121.0
Smoker				
1	105.0	102.0	180.0	175.0
2	102.0	99.0	176.0	186.0
3	100.0	104.0	182.0	182.0

3.3.12 Regeneration, reproducibility, stability and selectivity of MWNT/EPPGE

It was found that the peak current of epinephrine and norepinephrine slightly decreases when the potential scan was successively repeated. Thus, the modified electrode surface was regenerated in 0.5 M phosphate buffer solution by applying a potential of − 200 mV for 60 s. It was found that MWNT/EPPGE could be easily regenerated by applying a negative potential in blank PBS and then improved voltammetric response was observed. The current response of fixed concentration (60×10^{-9} M) of epinephrine and norepinephrine was measured individually for eight repetitive scans and the relative standard deviation of peak current was calculated to be 1.84% and 2.24%, respectively indicates that the modified electrode possesses satisfactory reproducibility.

The modified electrode retained 96% of its initial responses after continuous use for 12 days which suggested that the modified electrode comprise passable stability. The influence of analogous catecholamine dopamine and major biomolecules like uric acid and ascorbic acid was also examined and it was found that these compounds do not interfere in the detection, which indicates the adequate selectivity of the modified electrode for epinephrine and norepinephrine determination.

3.4 CONCLUSIONS

In this chapter, a new strategy using MWNT/EPPGE as a sensitive sensor for the assay of catecholamines – epinephrine and norepinephrine in human body fluids is described. EP and NE have been determined in plasma and urine samples of smokers and athletes for the first time by using voltammetric method. The modified electrode displayed strong catalytic function towards the oxidation of EP and NE leading to an increase in current response and a shift of the oxidation potential to lower values as compared to bare electrode. Modified electrode also resolved overlapped voltammetric response of both catecholamines into two well-separated peaks. Another imperative advantage of the developed sensor is that it can be used to monitor simultaneously different biomolecules at the electrode surface. Ascorbic acid, dopamine and uric acid showed oxidation peaks at –50, 80 and 260 mV, respectively which do not interfere with the oxidation of EP and NE confirming thereby that this voltammetric sensor is specific for the oxidation of EP and NE at 150 and 204 mV, respectively. To overcome major interference of uric acid and ascorbic acid, a specific potential region was selected by using MWNT/EPPGE to monitor EP and NE. In this region high concentration of uric acid and ascorbic acid resulting larger peaks did not suppress smaller peaks of catecholamines. EP and NE both involve catechol redox centre and difference of ~ 60 mV at modified electrode clearly indicates that the side chain plays an important role. Thus, protonation of primary amino group in NE and secondary amino group in EP at pH 7.2 seems to play a significant role. The proposed voltammetric method offers several advantages over the methods described previously for the determination of EP [29-40], including fast response, high sensitivity, low detection limit, adequate stability, and successful determination of catecholamines in body fluids even in the presence of high concentrations of uric acid and ascorbic acid. A comparison of the results with HPLC indicates that proposed method has adequate accuracy and the results are comparable. 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. Owing to its ample stability, reproducibility, selectivity and sensitivity the modified electrode could provide a promising tool for the individual and simultaneous determination of EP and NE in complex biological samples. Hence, the method is believed to be of beneficial use in clinical diagnosis, pharmaceuticals research, doping, smoking and nerve physiology.

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Chapter 4

DETERMINATION OF SOME IMPORTANT DRUGS/DOPING AGENTS AT SWNT/EPPGE

4.1 INTRODUCTION

Carbon nanotubes (CNTs) have attracted substantial interest as electrode surface modifiers due to their potential utility in fabricating highly sensitive nanoscale electrochemical sensors by facilitating the electron transfer and enhancing the electrode conductivity, thus improving the analytical sensitivity and selectivity [1, 2]. In general, two types of CNT materials are known, i.e., single-walled (SWNT) and multi-walled carbon nanotubes (MWNT) which consist of single and several graphite layers in the wall of a tube, respectively [3-5]. The lengths of CNTs are usually in the micrometer range with diameters from 0.4 to 3 nm for SWNT and from 1.4 to 100 nm for MWNT. Mainly the interest has been focused on the use of either type of CNT, namely SWNT or MWNT for development of advanced nanosensors. Literature survey reveals that very little information is available regarding the difference in electrocatalytic activity of two types of carbon nanotubes. In the proposed work efforts have been made to compare the electrocatalytic activity of two types of CNTs towards electrochemical determinations. A detailed comparison has been made between the response of SWNT and MWNT modified edge plane pyrolytic graphite electrode (EPPGE) in respects of several imperative analytical parameters viz. sensitivity, selectivity and detection limit in order to noticeably understand the difference in electrocatalytic activity of two types of carbon nanotubes.

Introduction of surfactants adds a beneficial and new dimension to electrode surface modification. The polar surfactants being amphiphilic molecules tend to adsorb at an interface or surface between electrode and solution through their polar head group and this phenomenon is advantageously used in electrochemistry. Due to the distinct amphiphilic structure and being surface active, surfactants are widely used in electrochemical investigations [6-9]. It is well documented that the modification of electrode surface by the surfactant increases electron transfer rate between the electrode surface and analyte [10, 11] and thus improves the detection limits of biomolecules [12, 13]. The exploit of surfactants for surface modification of various substrates has been reported previously [14-16] hence, owing to their beneficial effects efforts have been made in proposed work to examine surfactant-nanotube composite film modified EPPGE for electrochemical determinations.

The present chapter is resolute to individual and simultaneous determination of some important drugs or doping agents using carbon nanotubes and surfactants modified edge plane pyrolytic graphite electrode. The chapter is devided into three sections – first section

deals with the voltammetric determination of a new potent calcium antagonist, amlodipine besylate (ADB), in human body fluids mainly focused on the comparison of electrocatalytic activity of MWNTs and SWNTs towards the oxidation of ADB. The second section describes the simultaneous determination of two important synthetic corticosteroids – prednisolone and prednisone in human body fluids and pharmaceutical preparations. The third section explain the use of surfactant nanotubes composite film modified edge plane pyrolytic graphite electrode for determination of betamethasone, a potent pharmaceutical ingredient and doping agent, in urine samples of pregnant women who are undergoing treatment with this drug so that the method can be used to determine betamethasone in doping cases and other clinical purposes.

Calcium ions are required to generate electrical activity for the contraction of cardiac and smooth muscle and conduction of nerve cell. Calcium antagonist is a drug that inhibits the entry of excess calcium into cells and prevents the mobilization of calcium from intracellular stores, resulting in relaxation of blood vessel walls and cardiac muscle for blood to flow more freely. This causes lowering of blood pressure thereby reducing oxygen demand in the heart and relieving anginal pain. Amlodipine besylate (ADB), 3-ethyl 5-methyl (4RS)-2-(2-aminoethoxy) methyl)-4-(2-chlorophenyl) -6-methyl-1, 4-dihydropyridine-3, 5-dicarboxylate benzene sulphonate is a relatively new potent long-acting calcium channel blocking agent [17-19]. Amlodipine is a third-generation dihydropyridine calcium antagonist which is used alone or in combination with other medications for treating high blood pressure, certain types of vasospastic angina, hypertension, cardiac arrhythmias, and coronary heart failure [20-22]. It is more effective than β-blockers for the variant angina because it selectively inhibits the arterial vascular smooth muscle cell proliferation resulting in prevention of the progressive narrowing of the arteries and prevents the coronary spasms resulting in increased blood flow with myocardial oxygen supply [23-25]. ADB is available in the market as bulk material, tablets, capsules and compounded capsules, hence, owing to its therapeutic importance it is important to have a rapid and simple analytical technique for the determination of amlodipine in pharmaceutical preparations and human body fluids.

Various methods including high-performance liquid chromatography (HPLC) [26, 27], liquid chromatography (LC) [28], high-performance thin layer chromatography [29], gas chromatography (GC) [30], capillary electrophoresis [31], flow injection analysis [32], enzyme-linked immunosorbent assay [33], spectrofluorometric [34] and spectrophotometric

methods [35, 36] have been used for the determination of amlodipine besylate in biofluids and pharmaceutical preparations. Recently, in order to obtain an effective separation and sensitive detection some coupled methods such as liquid chromatography coupled with tandem mass spectrometry [37, 38] and solid-phase extraction with cation-exchange column [39] have also been used to monitor the ADB concentration. Several of the spectrophotometric procedures published show disadvantages such as a heating or extraction step, narrow range of linear response and also need of special reagents. Chromatographic methods offer the required sensitivity and selectivity but need sample clean-up, complicated extraction, tedious and time-consuming derivatization procedures, relatively heavy and expensive instrumentation which limited their use in quality control laboratories for analysis of amlodipine in its pharmaceutical dosage forms and human body fluids. Voltammetric methods have been found highly sensitive, convenient and effective tool for the analysis of important biomolecules including drugs in pharmaceutical formulations and human body fluids owing to their simplicity, low cost and relatively short analysis time as compared to the other routine analytical techniques. Hence, voltammetric determination of amlodipine using carbon paste electrode [40] and glassy carbon electrode [41-43] has also been reported; however, real samples analysis has not been performed. The proposed work using SWNT/EPPGE provides a simple, sensitive and rapid voltammetric method for the analysis of amlodipine in human body fluids and pharmaceutical products.

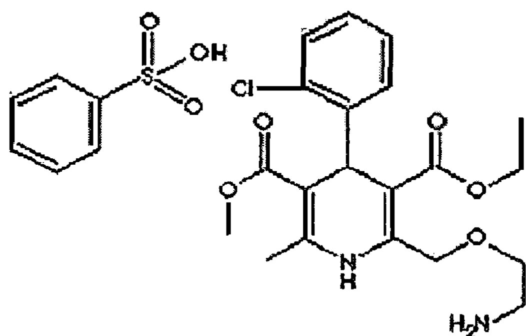
Corticosteroids affect biochemical events and cellular processes in tissues and organ of the body, hence, play a crucial role in human physiology [44]. Synthetic corticosteroids also comprise vital physiological activities, such as anti- inflammatory and anti-stress action and are regarded as the most effective treatment for topical diseases [45, 46] and in addition these drugs have the potential positive effects on sports performance. Hence, synthetic corticosteroids are extensively abused by athletes in competitive games and their use has been forbidden by the International Olympic Committee and World Anti Doping Agency. [47–49]. Prednisone and prednisolone are synthetic corticosteroids usually prescribed in the treatment of a wide variety of inflammatory diseases such as asthma, rheumatoid arthritis, various kidney diseases including nephritic syndrome, allergies and cluster headache [50-54]. These are available in market in the form of tablets, capsules, injections, ointments and creams and the use of both the compounds is banned in sports under antidoping rules [55-57]. Simultaneous determination of prednisolone and prednisone has great significance to bioscience and clinical diagnosis since prednisone is a biologically inactive 11- dehydro

metabolite of prednisolone. In human system prednisone is converted to the bioactive moiety prednisolone, via reduction of the 11-oxo group by the liver enzyme, 11-β-hydroxydehydrogenase [58, 59]. In mammals including humans interconversion of prednisone to prednisolone was found after oral administration of either of them with somewhat favoured prednisolone [60-62]. In view of the clinical importance and increased abuse of prednisolone and prednisone by athletes, it is considered desirable to analyze their concentrations in body fluids as well as in pharmaceutical formulations.

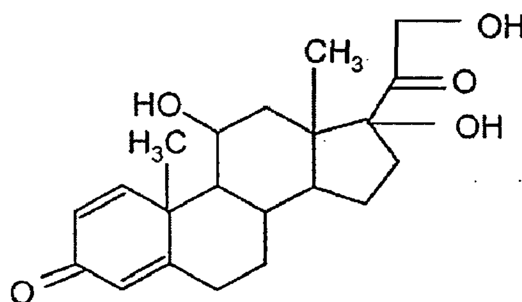
In view of the importance of corticosteroids in human physiology many methods such as spectroscopy and chromatographic methods coupled with spectroscopy or diode array detector and chemiluminescence have been developed for the simultaneous or individual determination of prednisone and prednisolone in biological fluids [63-66]. Polarography has also been attempted for determination of prednisone and prednisolone, however, the two peaks were found to be seriously overlapping [67]. In view of the increasing cases of doping by the athletes, simple, efficient, sensitive and reproducible analytical technique is needed to detect such cases at the site of games. Square wave voltammetry has been used for the sensitive determination of large number of anabolic steroids [68, 69]. However, the determination in these studies is based on oxidation of steroids. Since, blood and urine possess uric acid, dopamine, xanthine and ascorbic acid, and a major problem is encountered due to interference of their oxidation signals with analyte signal. Hence, in the present method attempts are focused on reduction based determination of prednisone and prednisolone in biological fluids using edge plane pyrolytic graphite electrode after modification with single wall carbon nanotubes.

Another corticoid betamethasone sodium phosphate; a disodium salt of the 21 – phosphate ester of betamethsone, is a potent pharmaceutical ingredient and finds widespread clinical applications related to anti-inflammatory and immunosuppressant activity [70]. It is well known to exert a growth promoting action, exalt the action of β-agonists and also act as an appetizer [71]. It has been extensively used in prenatal medicine to accelerate advance lung maturation in fetus of pregnant woman at risk of premature delivery [72, 73]. Betamethasone is also regarded as a standard treatment in premature infants to decrease the mortality incidences from intracranial hemorrhage [5, 74]. Being a glucocorticosteroids, betamethasone is highly lipophilic and can readily cross the placenta, but during the whole course of pregnancy the level of circulating drug is comparatively high in mother's body fluids, hence, it might be possible to detect its concentration in blood

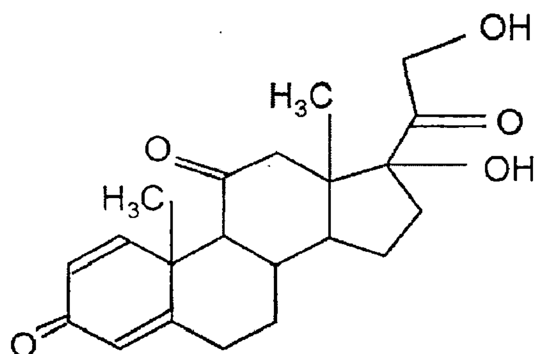
plasma or urine samples of pregnant woman. The anti-inflammatory action makes corticosteroids one of the most effective groups of drugs; however, they are one of the most abused classes of drugs also. Together with the already described effects betamethasone also affects nervous system (euphoria), carbohydrate metabolism (stimulation of gluconeogenesis), mobilization of free fatty acids, catabolism of proteins, cardiovascular apparatus, and blood (increase of hemoglobin and red cell content) which make this drug attractive for doping purposes [75, 76]. Illicit use of betamethasone in zootechniqes has also been reported. Inspite of extensive use and misuse of betamethasone, very little information is available in literature about the determination of this drug in human body fluids as indicated by lesser number of publications available [77-79]. Techniques used in available literature such as spectrometry and chromatography required lengthy and tedious procedures viz. sample preparation, purification and extraction which are undoubtedly critical steps in corticosteroids analysis. Hence, there is an expanding demand for the development of simple, reliable, rapid and efficient sensors with enhanced characteristics for effective sensing of betamethasone in biofluids in order both to monitor clinical administration and to verify compliance with the law. Thus, the objective of the present study is to develop an efficient sensor using surfactant for the determination of betamethasone in urine samples of pregnant women who are undergoing treatment with this drug so that method can also be employed to determine betamethasone and analogous drugs in doping cases. Thus, in this chapter results on the studies of amlodipine besylate, prednisone, prednisolone and betamethasone are presented.



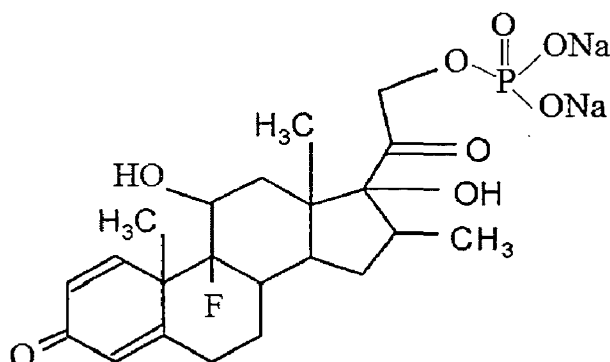
Amlodipine Besylate



Prednisolone



Prednisone



Betamethasone sodium phosphate

4.2 EXPERIMENTAL

4.2.1 Instrumentation

Electrochemical experiments were performed with a computerized BAS (Bioanalytical Systems, West Lafayette, USA) CV-50W electroanalyzer. A regular three-electrode system consisting of a bare or nanotubes and surfactant modified edge plane pyrolytic graphite working electrode, platinum-wire auxiliary electrode and an Ag/AgCl reference electrode (3 M NaCl) (Model MF-2052 RB-5B) was used. The surface morphology of bare and modified edge plane pyrolytic graphite electrodes was characterized using JEOL JSM-7400F field emission scanning electron microscopy (FE-SEM) instrument. The pH of the buffer solutions was measured using Century India Ltd. Digital pH-meter (Model CP-901) after standardization. Ultrasonic machine was used to achieve well dispersed suspension of nanotubes in N, N-dimethylformamide (DMF) and cetyltrimethyl ammonium bromide (CTAB) solutions. High performance liquid chromatography (HPLC) experiments were performed on Agilent 1100 series system with reverse phase column RP- 18e (5 μM).

4.2.2 Reagents and standards

Amlodipine besylate was obtained from Ish Medicos Private Limited, Dehradun. Prednisone and prednisolone were obtained from Sigma (St. Louis, MO, USA). Prednisolone, amlodipine and betamethasone containing tablets manufactured by different pharmaceutical companies were purchased from the local market of Roorkee.

Betamethasone disodium phosphate for voltammetric investigations was obtained as a gift from Cito Chem (P) Ltd. Indore (MP) India. The multi- and single- walled carbon nanotubes (purity SWNT >98 %, MWNT >98%) were purchased from Bucky, USA. Pyrolytic graphite pieces were obtained as a gift from Pfizer USA. Phosphate buffer solutions (PBS) of desired pH and ionic strength were prepared by mixing the stock solutions of analytical grade Na_2HPO_4 and NaH_2PO_4 according to the method of Christian and Purdy [80] using analytical grade chemicals from Merck. Control urine sample were received from healthy laboratory personnel and urine and blood plasma samples of patients undergoing treatment with amlodipine, prednisolone and betamethasone were obtained from the Institute hospital of IIT Roorkee. Doubly distilled water was used throughout the experiment. All other reagents and solvents were of analytical grade and used as received.

4.2.3 Recommended procedure

The stock solution of ADB (1 mmol L^{-1}) was prepared in methanol/water (1:10). The stock solutions of desired concentration of prednisone and prednisolone were prepared in methanol (A.R.). Stock solution of betamethasone sodium phosphate (1 mmol L^{-1}) was prepared in double distilled water for voltammetric measurements. The solution was deoxygenated by bubbling nitrogen for 8-10 min. before recording the voltammograms for reduction studies. Working solutions were prepared by diluting stock solution with 2 mL of phosphate buffer solution. All the potentials are referred to Ag/AgCl electrode at an ambient temperature of 25 ± 2 °C. Square wave voltammetric parameters; step E = 4 mV, square wave amplitude (E_{SW}) = 25 mV, square wave frequency (f) = 15 Hz and quiet time 2 s were selected as the optimum. The mobile phase used for HPLC experiments was a mixture of water: methanol (60:40) at a flow rate of 1.4 ml min^{-1} and absorbance of the eluent was monitored at 254 nm.

4.2.4 Preparation of SWNT/EPPGE

The bare surface of EPPGE was rubbed on an emery paper and then washed with double distilled water prior to the preparation of nanotubes modified EPPGE. The SWNT suspension was prepared by dispersing 0.5 mg SWNT in 1 mL N, N-dimethylformamide (DMF) with the aid of ultrasonic agitation for 30 min. The modified electrode was then prepared by casting different amount of solution of carbon nanotube. It was found that the current was maximum at 40 μL solution. Hence, 40 μL of the above suspension of SWNT

onto the surface of bare EPPGE was casted and then the solvent was allowed to evaporate. The MWNT modified EPPGE was prepared with the same procedure as mentioned above for the SWNT/EPPGE. The modified electrode surface was cleaned after each run by immersion into blank PBS and applying a potential of + 200 mV (for reduction studies) for 60 s to desorb the adsorbed material. The modified electrodes were stored in air at room temperature. The surface of modified electrodes was characterized using field emission scanning electron microscopy.

4.2.5 Preparation of SWNTs-CTAB/EPPGE

Cetyltrimethyl ammonium bromide (5 mg) was dissolved in 1 mL double distilled water and then 0.5 mg SWNT material was dispersed in 1 mL CTAB solution. To achieve well dispersed suspension of nanotubes in cetyltrimethyl ammonium bromide solution the mixture was agitated for an hour using ultrasonic bath. The beneficial influence of CTAB has been reported to be restricted at its optimum concentration because surfactant molecules adsorbed on the electrode surface at low concentration and a layer is formed on electrode surface, but at high concentration of surfactants, due to the micelle formation the charge transfer is affected in opposite manner which results in poor modifications [81]. The poor modification at high concentrations of surfactant has been confirmed by the poor voltammetric response in many cases [81]. Therefore, 5 mg of CTAB was dissolved in 1 mL double distilled water to achieve optimum concentration. To remove adhered particles the surface of edge plane PGE was rubbed on an emery paper and washed with double distilled water and then touched softly onto tissue paper. 30 μL of well dispersed nanotubes suspension in CTAB solution was adsorbed onto the electrode surface and then dried at room temperature. Adsorbed compound from the surface of modified electrodes has been removed by applying a potential of + 200 mV (for reduction study) for 60 s. The surface of modified electrodes was characterized using field emission scanning electron microscopy (FE-SEM). A comparison of typical FE-SEM images of (a) bare EPPGE, (b) multi-walled carbon nanotubes-dimethyl formamide film modified modified EPPGE, (c) single walled carbon nanotubes-dimethyl formamide film modified modified EPPGE and (d) single walled carbon nanotubes-cetyltrimethylammonium bromide nanocomposite film modified edge plane pyrolytic graphite electrode (SWNTs-CTAB/EPPGE) is presented in **Fig 4.1**, which clearly indicates the deposition of different kind of modifiers on the surface of bare EPPGE.

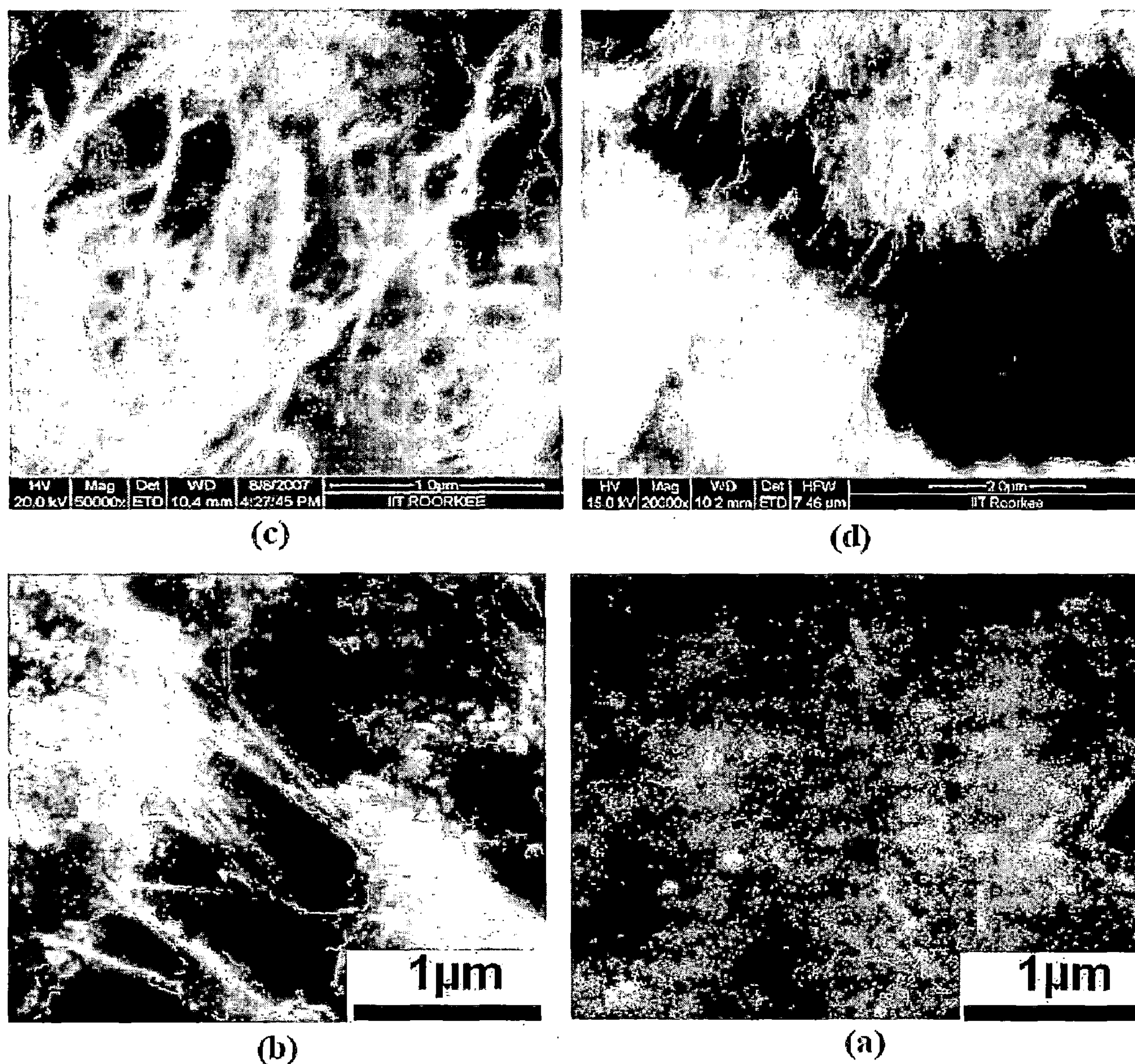


Fig. 4.1 FE-SEM images of (a) bare EPPGE, (b) MWNT/EPPGE, (c) SWNT/EPPGE and (d) SWNTs-CTAB/EPPGE.

4.2.6 Surface area

One of the potential regions for the enhanced electrocatalytic properties of electrodes is larger surface area. Cyclic voltammograms of 1mM $K_3Fe(CN)_6$ containing 0.1 M KCl were recorded at different scan rates using bare EPPGE, MWNT/EPPGE, SWNT/EPPGE and SWNTs-CTAB/EPPGE in order to calculate surface area. A redox

couple was observed due to the Fe^{+3}/ Fe^{+2} at bare and both the modified electrodes and corresponding reversible processes follow the equation:

$$i_p = 0.4463\ (F^3/RT)^{1/2} A\ n^{3/2}\ D_R^{1/2}\ C_0\ \nu^{1/2}$$

where F is Faraday's constant (96485 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 (Ampere), n = 1 for $K_3Fe(CN)_6$, T is the absolute temperature (298 K), $D_R = 7.6 \times 10^{-6}$ cm^2 s^{-1}, ν is scan rate (Vs^{-1}) and C_0 is the concentration of $K_3Fe(CN)_6$ in $molL^{-1}$. The surface area was calculated from the slopes of the i_p versus $\nu^{1/2}$ plots and found as 0.0675 and 0.1253 cm^2 for MWNT/EPPGE and SWNT/EPPGE, respectively. Similarly, the surface areas of bare and SWNTs-CTAB/EPPGE were found as 0.0476 and 0.1929 cm^2, respectively. Experimental results thus indicate that the surface area of MWNT/EPPGE and SWNT/EPPGE is ~1.4 and 2.6 -fold larger than the surface area of the bare EPPGE and the surface area of SWNTs-CTAB/EPPGE is almost four times larger than bare edge plane pyrolytic graphite electrode.

4.3 RESULTS AND DISCUSSION

(A) AMLODIPINE BESYLATE

4.3.1 Electrochemical behavior of amlodipine

4.3.1.1 Cyclic voltammetry

Cyclic voltammograms were recorded for 5 μM amlodipine in 1 M PBS solutions (pH 7.2) using bare, MWNT and SWNT modified EPPGE at scan rate 50 mV/s. With the bare EPPGE, a broad oxidation peak (small bump) at peak potential ~ 700 mV with very low current value indicates that the bare EPPGE shows very poor response towards the amlodipine oxidation. After modification with multi walled and single walled nanotubes a well-defined peak appeared at anodic peak potentials of ~ 605 mV and ~ 524 mV, respectively for amlodipine oxidation with remarkable enhancement in peak current values as compared to bare pyrolytic graphite electrode. Absence of reduction peak on reverse sweep clearly indicates that amlodipine is irreversibly oxidized at CNT modified EPPGE. The negative-shift of peak potential and the marked enhancement in the anodic peak current of amlodipine oxidation confirm the electrocatalytic effects of the carbon nanotubes towards the oxidation of amlodipine. **Fig. 4.2** compares cyclic voltammograms for 5 μM ADB using MWNT (curve a) and SWNT (curve b) modified edge plane pyrolytic graphite electrodes

recorded at 50 mV/s and clearly indicates that SWNT/EPPGE serves as a better sensor in comparison to MWNT/EPPGE.

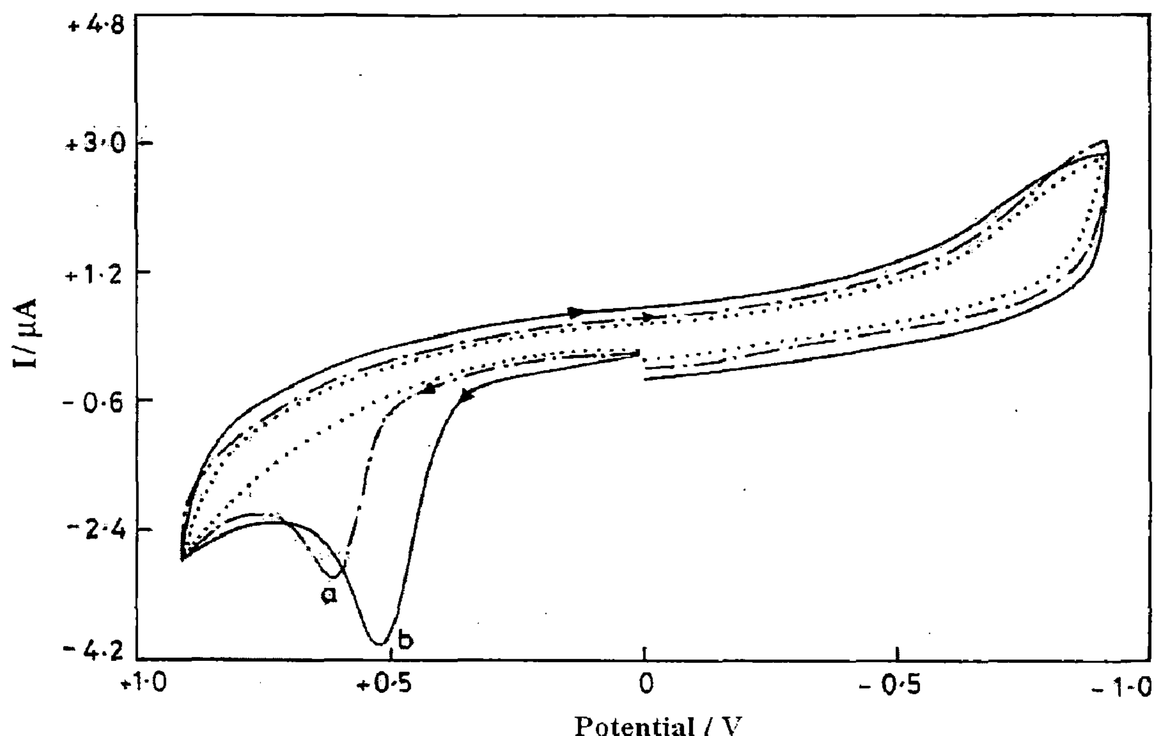


Fig. 4.2 Cyclic voltammograms of 5 µM amlodipine using MWNT modified EPPGE (a), SWNT modified EPPGE (b) and dotted CV is response of blank PBS of pH 7.2 using SWNT/EPPGE at scan rate 50 mV/s.

4.3.1.2 Effect of scan rate

The effect of scan rate on the oxidation of amlodipine was examined in the range 20 to 1000 mV/s at pH 7.2 using SWNT modified EPPGE by cyclic voltammetry as shown in **Fig. 4.3**. The peak current of 5 µM amlodipine solution at the SWNT/EPPGE increased linearly with increase in scan rate and this behaviour indicates the direct electron transfer on the surface of the modified electrode and ADB. **Fig. 4.3 (inset A)** presents a linear plot between peak current and scan rate having correlation coefficients of 0.9972. The linear relation between i_p and ν can be represented by the equation:

$$i_p / \mu A = 0.0475 \ (\nu / mV \ s^{-1}) + 1.7142$$

The oxidation peak potential also increased with scan rate and the plot of E_p versus log v **{Fig. 4.3 (inset B)}** was linear. The dependence of E_p can be expressed by the equation:

$$E_p / mV = 79.394 \; (\log v / mV\, s^{-1}) + 387.39$$

having correlation coefficient 0.9967. These observations suggest that the adsorption plays a significant role [82-84] in the irreversible electrode reaction of ADB at SWNT modified EPPGE.

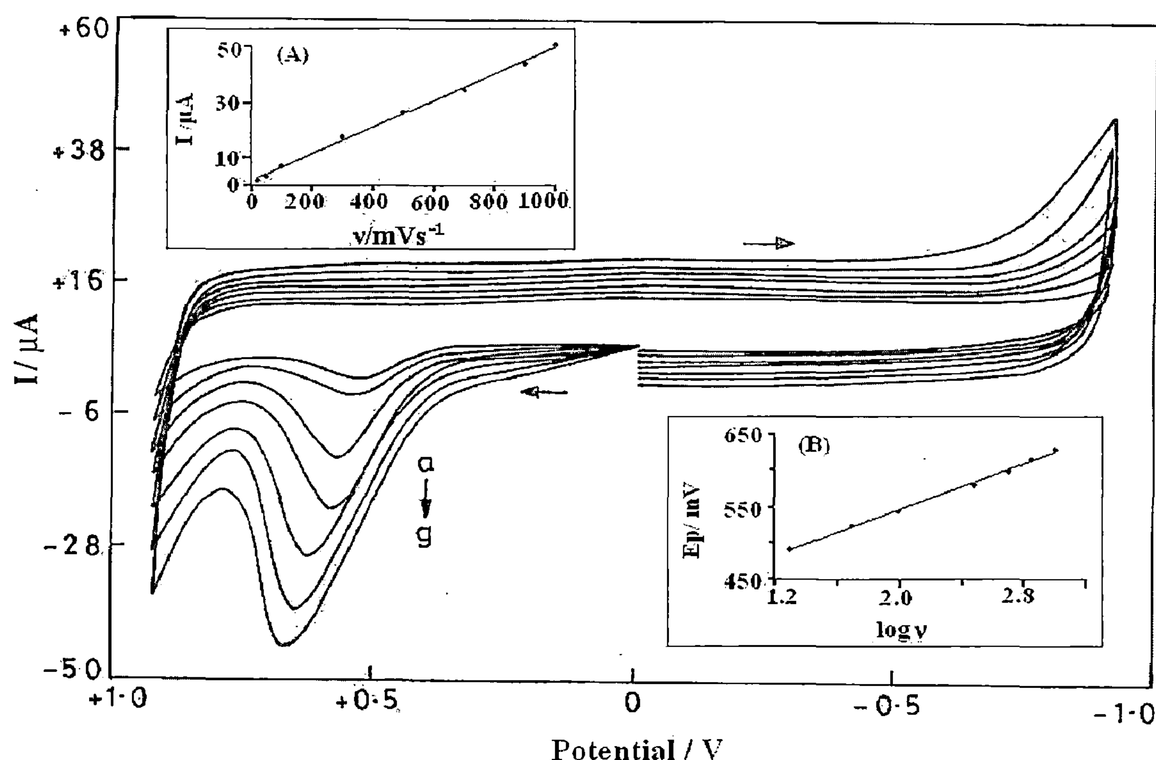


Fig. 4.3 Cyclic voltammograms obtained at different scan rates using SWNT modified EPPGE in PBS of pH 7.2 containing 5 µM amlodipine; Scan rates: 50, 100, 300, 500, 700, 900 and 1000 mV/s (a-g, respectively); Inset (A) plot of anodic peak currents as a function of scan rate and Inset (B) plot of peak potential as a function of scan rate.

4.3.1.3 Square wave voltammetry

The electrochemical behaviour of amlodipine was also studied by square wave voltammetry using bare EPPGE, MWNT/EPPGE and SWNT/EPPGE for the purpose of determination. **Fig. 4.4** depicts the electrochemical response of 1.0×10^{-6} mol L^{-1} amlodipine under optimum parameters in phosphate buffer of pH 7.2 using above mentioned three electrodes. A small oxidation peak is observed at the bare EPPGE having peak potential ~ 634 mV (curve a). At MWNT/EPPGE amlodipine gets oxidized at ~ 532 mV (curve b) whereas at SWNT modified EPPGE the oxidation peak potential shifts to ~ 456 mV (curve c) with enhancement in the peak current. A comparative study using square wave voltammetry clearly indicates that a substantial decrease (178 mV and 102 mV) in the E_p of the ADB oxidation reaction (compared to bare surface of EPPGE) is observed using single-walled and multi-walled carbon-nanotubes coatings, respectively. The peak currents are ~2.2 (b) and 4.0 c-folds larger than the corresponding peak (a) at the bare surface. The decrease of anodic peak potential and enhancement of peak current indicate that CNTs exhibits efficient electrocatalysis towards amlodipine oxidation and thereby improving the electrochemical response. It is also concluded that single-walled carbon nanotubes exhibit enhanced catalytic effects as it accelerates the rate of electron transfer faster towards the oxidation of amlodipine as compared to multi-walled carbon nanotubes. Therefore, it is concluded that SWNT appear to be a better electrode surface modifier as compared to MWNT for the voltammetric oxidation of amlodipine. Since square wave voltammetry is more sensitive technique than cyclic voltammetry, hence, detailed analysis of amlodipine is performed utilizing square wave voltammetry.

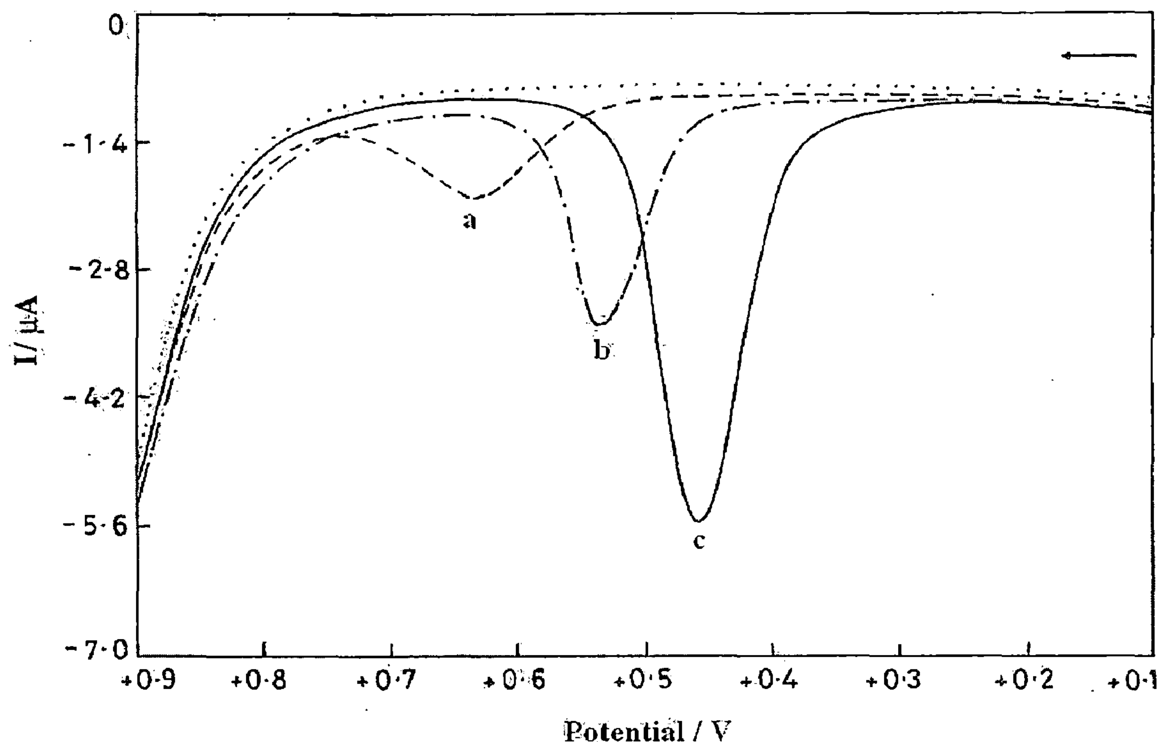


Fig. 4.4 Comparison of square wave voltammograms of 1 μM amlodipine at pH 7.2 using bare EPPGE (a), MWNT/EPPGE (b), SWNT/EPPGE (c) and dotted line indicates the response of blank PBS at SWNT/EPPGE.

4.3.2 Effect of pH

The electrochemical behavior of amlodipine was investigated using phosphate buffer in the pH range 2 to 11. The influence of pH on the oxidation peak of amlodipine was examined using SWNT/EPPGE and MWNT/EPPGE by square wave voltammetry. It is observed that with increase in pH the peak potential shifts towards less positive values, which indicates the participation of protons in the electrode process. The slope of the linear E_p vs. pH equation was ~ 58 mV and suggested that equal number of protons and electrons are involved in the electrode reaction at both the modified electrodes. The linear relationship between the peak potential (E_p) and pH using MWNT and SWNT modified EPPGE can be expressed by the following equations having correlation coefficient 0.9852 and 0.9916, respectively.

E_p / mV = 951.3 – 57.38 pH at MWNT/EPPGE

E_p / mV = 879.91 – 58.09 pH at SWNT/EPPGE

4.3.3 Detection limit and sensitivity

The variation of oxidation peak current with ADB concentration was studied using both multi- and single walled nanotubes modified EPPGE. Various concentrations of amlodipine were prepared within range 5.0×10^{-9} to 1.2×10^{-6} $molL^{-1}$ and square wave voltammograms were recorded. **Fig. 4.5** illustrates a series of square wave voltammograms obtained for ADB at different concentrations using SWNT/EPPGE in 1 M PBS solution at pH 7.2. When the concentration (C) gradually increases the oxidation peak current (i_p) linearly increases at both the modified electrodes as shown in **Fig. 4.6**. The dependence between the peak current (i_p) and concentration can be expressed by the following equations:

$i_p / \mu A = 0.0026$ ([Amlodipine]/nM) + 0.0021 at MWNT/EPPGE

$i_p / \mu A = 0.0047$ ([Amlodipine]/nM) + 0.0005 at SWNT/EPPGE

having correlation coefficients 0.9979 and 0.9981, respectively. The detection limit was calculated by using the relation $3\sigma / b$, where σ is standard deviation of the blank and b is slope of the calibration curve and found to be 5 nM and 1 nM for MWNT and SWNT modified electrodes, respectively. The observed sensitivities at MWNT and SWNT modified EPPGE are 0.0026 and 0.0047 $\mu A\ nM^{-1}$, respectively as shown by the above mentioned regression equations; indicating thereby that the detection sensitivity at SWNT modified EPPGE is about 1.8 times more than at MWNT modified EPPGE. Therefore, it can be concluded that SWNT/EPPGE is comparatively a better electrode towards the oxidation of amlodipine as compared to MWNT/EPPGE in terms of important analytical parameters such as detection limit, sensitivity, and peak potential and current. Owing to comparatively better voltammetric response at SWNT/EPPGE it is decided to use it for further detailed analysis of amlodipine in biological and pharmaceutical samples.

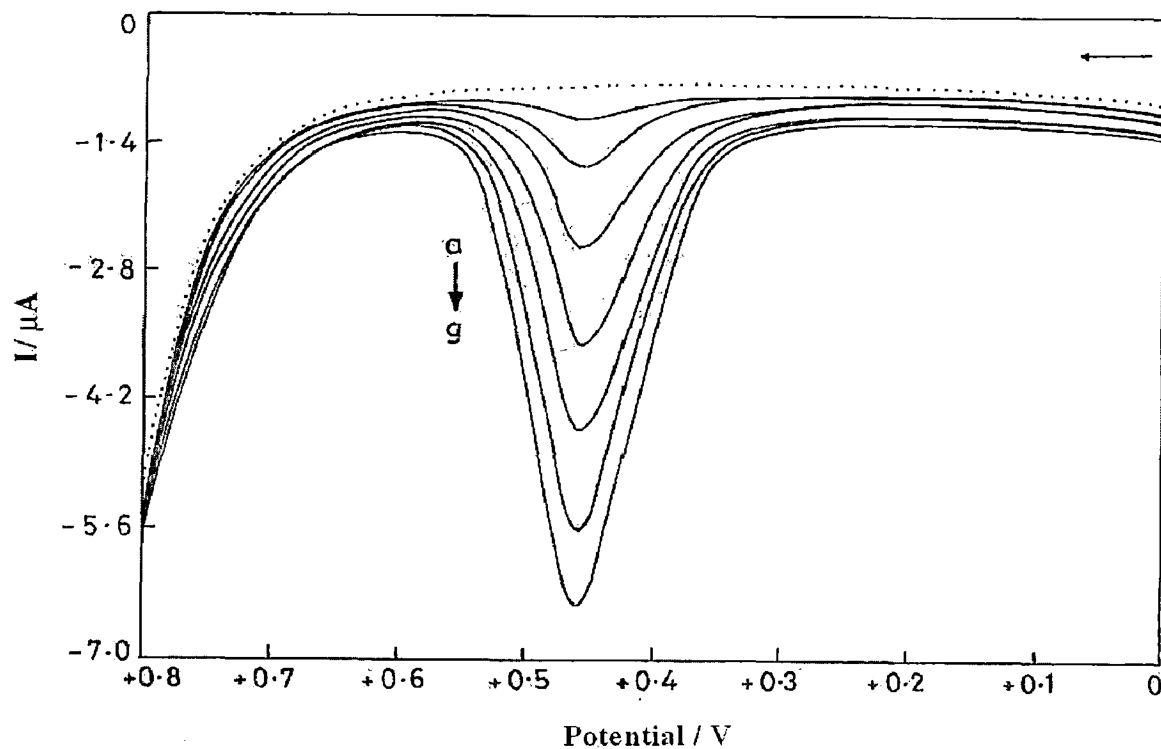


Fig. 4.5 Square wave voltammograms for 0.05, 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 µM ADB (a-g, respectively) and dotted line indicates the response of blank PBS of pH 7.20 at SWNT/EPPGE.

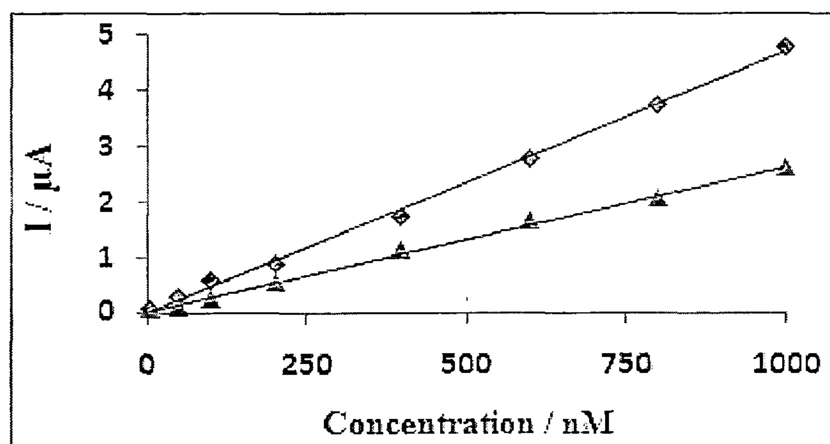


Fig. 4.6 Calibration plots observed for amlodipine using MWNT modified EPPGE (▲) and SWNT modified EPPGE (■) at pH 7.2.

4.3.4 Real sample analysis

4.3.4.1 Determination of amlodipine in human urine

The applicability of the proposed method was examined on human urine samples obtained from angina patients undergoing treatment with amlodipine. Urine sample of healthy volunteer was used as control. Amlodipine has the longest half-life (30-50 h) of elimination in humans among all the drugs of its class [85] therefore, it is expected that sufficient amlodipine is metabolized in about 24 h. Hence, urine samples of patients were received for analysis after 24 h of administration of single dose of 5 mg Amdepin-5 tablet. The anthropometric data of the patients were, Sample 1: male, age 54 yrs, height 165 cm, weight 65 kg; Sample 2: female, age 42 yrs, height 154 cm, weight 55 kg and Sample 3: female, age 45 yrs, height 160 cm, weight 62 kg. The samples were used for analysis after 10 times dilution with phosphate buffer solution (pH 7.2) to minimize matrix complexity and square wave voltammograms were recorded using SWNT/EPPGE. **Fig. 4.7** (a) presents a typical square wave voltammogram observed for the control urine sample from healthy volunteer and exhibited peaks at ~ 200 mV, ~ 300 mV and ~ 600 mV. The urine sample 1 collected from angina patient exhibited a well defined peak ~ 456 mV corresponding to amlodipine along with additional peaks at ~ 200 mV, ~ 300 mV and ~ 600 mV (curve b). To confirm that peak at ~ 456 mV is due to amlodipine, voltammograms were also recorded after spiking the urine sample 1 with 0.5 μM amlodipine. On spiking amlodipine, the peak height of the peak at 456 mV increases which confirm that peak at ~ 456 mV is due to the oxidation of amlodipine. The additional peaks in urine samples are due to the presence of major endogenous metabolites ascorbic acid, uric acid and xanthine, which were characterized using the standard compounds. Keeping dilution factor in consideration the concentration of amlodipine in the human urine of patient (Sample 1) was found to be 0.5 μM using the regression equation. To reconfirm the results urine samples of three patients were spiked with known concentrations of standard amlodipine. The results obtained for urine samples of three different patients are tabulated in **Table 4.1** and indicate that after 24 h of oral administration amlodipine is excreted in urine in about 0.50 μM concentration.

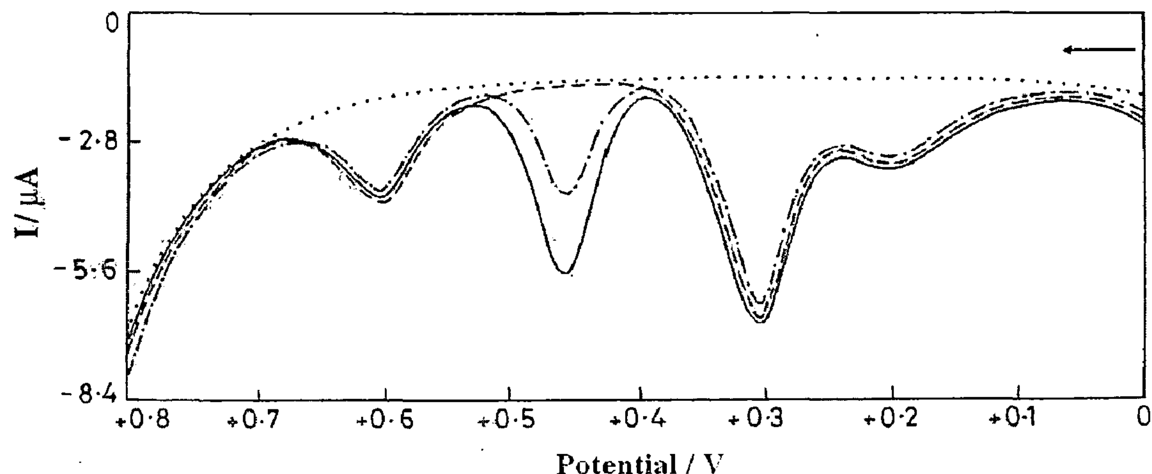


Fig. 4.7 Square wave voltammograms for urine sample of healthy volunteer (- - - -), urine sample 1 of angina patient (–·–·–·) and sample 1 of patient spiked with 0.5 µM amlodipine (——) and, dotted line indicates the response of blank PBS of pH 7.20 at SWNT/EPPGE.

Table 4.1 Analysis of amlodipine in urine samples of angina patients using SWNT/EPPGE

Sample	Spiked (µM)	Detected* (µM)	Actual Concentration (µM)
1	0.0	0.50	0.50
	0.50	1.01	0.51
	1.00	1.99	0.49
2	0.0	0.52	0.52
	0.50	1.04	0.54
	1.00	2.06	0.56
3	0.0	0.48	0.48
	0.50	0.95	0.45
	1.00	1.96	0.46

*The R.S.D. value for determination was less than 2.8 % for $n = 3$.

4.3.4.2 Quantification of amlodipine in doses forms

In order to evaluate the validity of the proposed method the content of amlodipine in the pharmaceutical medicinal tablets was also determined. Tablets containing amlodipine viz. Amtas-5 (INTAS Pharmaceuticals, Mfg. Lic. No. : 15/UA/2007), Amlopres – 5 (Cipla Limited, Mfg. Lic. No.: M/447/2007), Asomex – 2.5 (Emcure[R] Pharmaceuticals Limited, Mfg. Lic. No.: JK/01/08-09/155), Amlovas–5 (Macleods Pharmaceuticals Ltd., Mfg. Lic. No. MNB/07/594), Amlopin –5 (USV Limited, Mfg. Lic. No.: DD/291), Amdepin–5 (CADILA Pharmaceuticals, Mfg. Lic. No.: JK/01/06-07/110) were purchased from the local market of Roorkee. Firstly amlodipine tablets were dissolved in methanol/water (1:10) solution. To achieve amlodipine concentration in the range of calibration plot tablets solutions were subsequently diluted and then square wave voltammograms were recorded under optimum conditions. The concentration of amlodipine determined using the proposed method is reported in **Table 4.2.** The RSD for three parallel detections is less than 3.6 % for n = 3.

Table 4.2 Analysis of pharmaceutical samples containing amlodipine using SWNT/EPPGE

Sample	Claimed amount (mg/tablet)	Detected amount (mg/tablet)	Error (%)
Amtas	5.0	4.9	– 2.0
Amlopres	5.0	4.8	– 4.0
Amlovas	5.0	4.8	– 4.0
Amlopin	5.0	4.9	– 2.0
Amdepin	5.0	4.9	– 2.0
Asomex	2.5	2.4	– 4.0

4.3.5 Interferences study

As the major metabolites present in human urine are uric acid, ascorbic acid and xanthine which can interfere in the detection of analyte because of being redox active

therefore, the possible interferences of these compounds on the oxidation peak of amlodipine have been evaluated to examine the selectivity of the proposed method. The experimental results show that upto1000-fold excess of uric acid, ascorbic acid, xanthine and hypoxanthine, they have no interference on the square wave voltammetric determination of 1×10^{-6} mol L^{-1} amlodipine using SWNT/EPPGE. The analysis of the obtained results indicates that these compounds do not interfere in the determination of amlodipine using the proposed method.

(B) PREDNISOLONE AND PREDNISONE

4.3.6 Electrochemical behaviour of prednisone and prednisolone

A comparison of typical square wave voltammograms of 10 μM of prednisolone and prednisone at the bare and modified edge plane pyrolytic graphite electrodes in phosphate buffer of pH 7.2 is presented in **Fig. 4.8**. Two peaks for the reduction of prednisone and prednisolone were observed at potentials ~ – 1299 and ~ –1412 mV, respectively at the bare electrode. The peak obtained for both the compounds is rather broad, suggesting slow electron transfer kinetics. However, with increase in concentration of prednisolone the second peak merged with background, hence, the attempt to use bare EPPGE for the determination of two steroids was not successful. Whereas at SWNT/EPPGE, two well-defined reduction peaks were obtained and the reduction peak potential of prednisone and prednisolone were shifted to less negative potentials, ~ –1230 and ~ –1332 mV, respectively and the peak current was also increased. The above results suggested that the SWNT modified electrode promoted the electrochemical reduction of prednisone and prednisolone by considerably accelerating the rate of electron transfer. One of the reasons for this catalysis is that metallic impurities in nanotubes act as a promoter by increasing the rate of electron transfer, thus, a negative shift in their reduction potentials is observed. The electrocatalytic activity of SWNT has been assigned by many workers to entrapped metals in the cavity [86, 87].

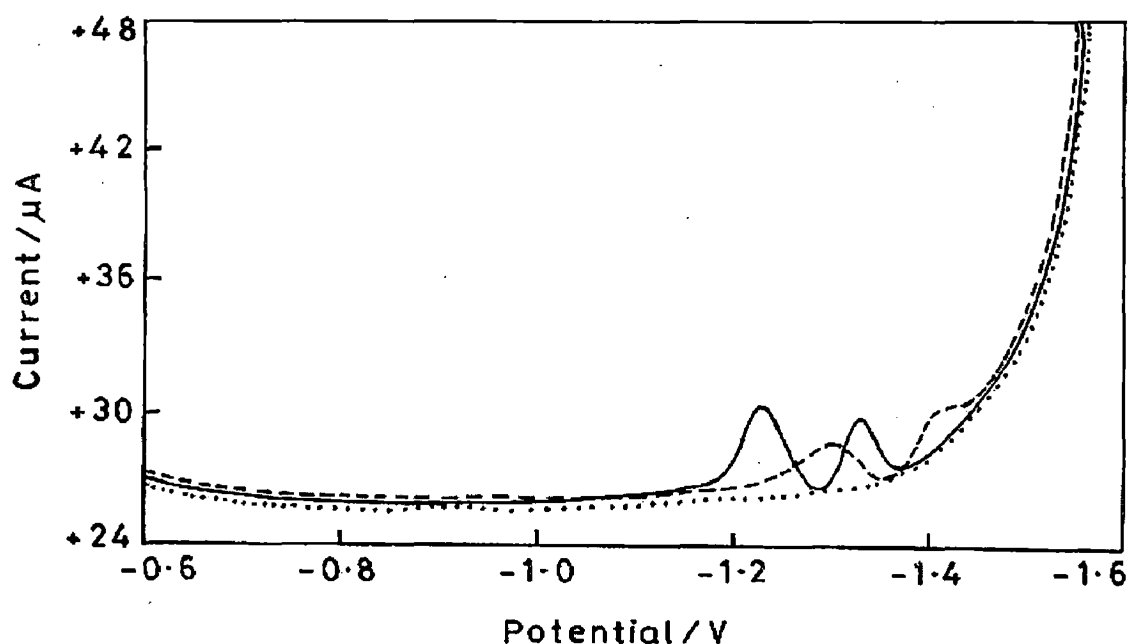


Fig. 4.8 Observed square wave voltammograms for the reduction of 10 μM of each prednisolone and prednisone at bare EPPGE (- - - -), SWNT/EPPGE (——) and background PBS (pH 7.2) at SWNT/EPPGE (……).

4.3.7 Effect of pH and square wave frequency

The effect of pH on the reduction of both the drugs was studied in the pH range 2.4 to 10.0. The peak potential (E_p) of both the drugs was found to shift towards more negative potentials with increase in pH {**Fig. 4.9 (A)**}. The variation of peak potential (E_p) with pH was linear for both the analytes and the dependence of E_p on pH obey the relations:

$- E_p$ (pH 2.4 – 10.0) = [825.52 + 54.53 pH] versus Ag / AgCl for prednisone

$- E_p$ (pH 2.4 – 10.0) = [944.52 + 54.58 pH] versus Ag / AgCl for prednisolone

having correlation coefficients 0.9973 and 0.9968, respectively. The slope of the $- E_p$ vs. pH curves was ~ 55 mV/pH for both the analytes, indicating that equal number of protons and electrons are involved in the electrode reaction.

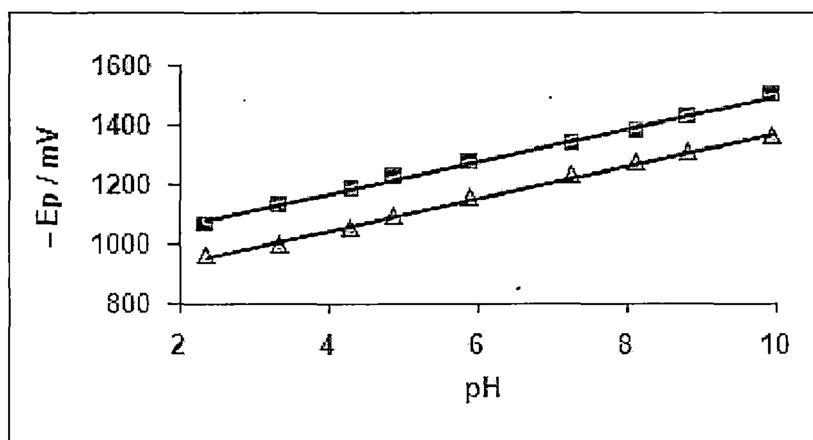


Fig. 4.9 (A) Observed dependence of peak potential (– E_p) on pH for 10 μM prednisone (▲) and 10 μM prednisolone (■) at SWNT modified EPPGE.

The influence of square wave frequency (f) on the peak current (i_p) of both analytes was examined in the range of 5 to 75 Hz. At frequency higher than 75 Hz, the peaks merged with the background. The peak current (i_p) of both the analytes was found to increase with increase in square wave frequency (f). The dependence of peak current on square wave frequency was linear suggesting thereby that the electrode reaction for both the compounds is adsorption controlled [84, 88]. The variation of i_p with f can be expressed by the equations:

$i_p\,(\mu A) = 0.2268 f + 0.4283$ for prednisone

$i_p\,(\mu A) = 0.2215 f + 0.1444$ for prednisolone

having correlation coefficients 0.9958 and 0.9991, respectively.

The peak potential of prednisone and prednisolone were also found to shift towards more negative potentials with increase in square wave frequency and the plots of E_p versus $\log f$ were linear in the frequency range 5-75 Hz {**Fig. 4.9 (B)**}. The variation of E_p with $\log f$ can be expressed by the equations:

$-E_p\,(mV) = 103.22 \log f + 1107.4$ for prednisone

$-E_p\,(mV) = 98.775 \log f + 1212.6$ for prednisolone

with correlation coefficients 0.9988 and 0.9929, respectively. Such a behavior indicates the nature of redox reaction as reversible [88].

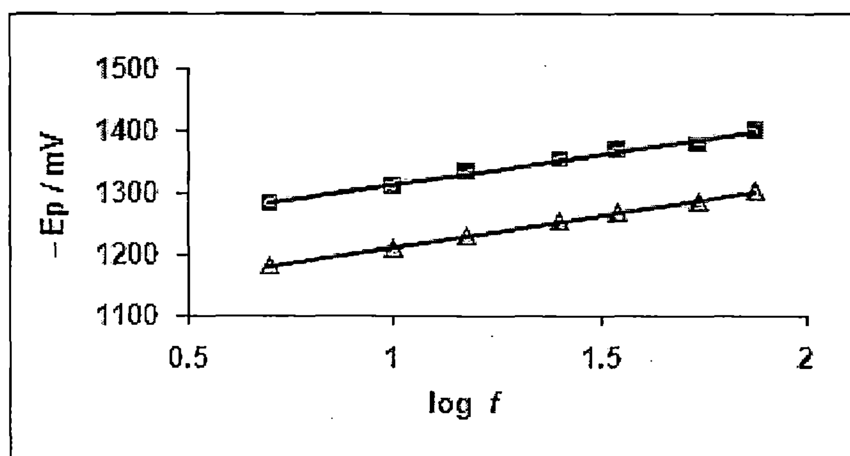


Fig. 4.9 (B) Plot of – E_p vs. logarithm of frequency (log *f*) of 10 µM prednisone (▲) and 10 µM prednisolone (■) at SWNT modified EPPGE.

4.3.8 Individual determination of prednisone and prednisolone

The square wave voltammograms were recorded for prednisone as well as prednisolone at different concentrations in the range 0.01 µM to 100 µM. It was found that the peak currents increased linearly with increasing concentration **{Fig.4.9 (C)}**. The graphs between peak current (i_p) and concentration from the data generated during SWV studies were linear and the relation can be represented by the equations:

i_p (µA) = 0.3233 C (µM) + 0.8415 for prednisone

i_p (µA) = 0.1897 C (µM) + 0.5815 for prednisolone

with correlation coefficients 0.9993 and 0.9945, respectively. The observed sensitivities for prednisone and prednisolone are 0.33 and 0.19 µA μM^{-1}, respectively indicating that both analytes can be safely estimated in the given concentration range. The detection limit of prednisone and prednisolone was calculated by using the formula 3σ / b, where σ is standard deviation of the blank and b is slope of the calibration curve, and found to be 0.45×10^{-8} and 0.90×10^{-8} M, respectively.

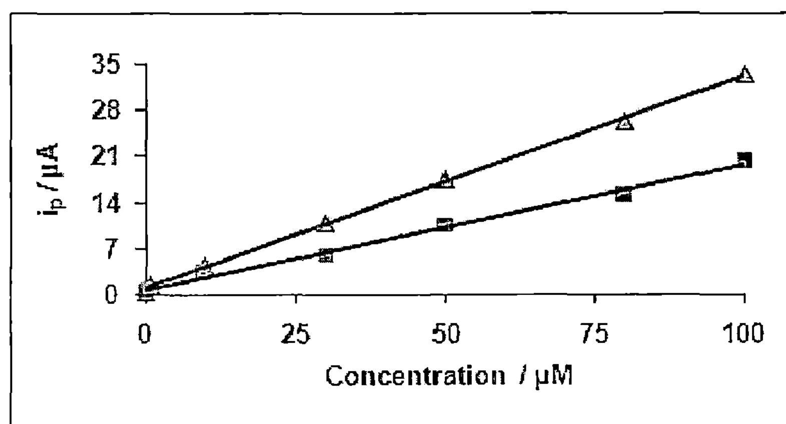


Fig. 4.9 (C) Calibration plots observed for prednisone (▲) and prednisolone (■) using SWNT modified EPPGE at pH 7.2.

4.3.9 Simultaneous determination of prednisone and prednisolone

The main aim of present study was to simultaneously investigate the electrochemical response of prednisone and prednisolone in human body fluids, as metabolism of prednisolone in human system proceeds through prednisone. The main problem encountered with simultaneous determination in earlier reports was close reduction potentials due to which it was difficult to resolve the overlapped voltammograms [89]. This problem was resolved in the present studies using SWNT modified edge plane pyrolytic graphite electrode. The simultaneous determination of prednisone and prednisolone at modified EPPGE was carried out by fixing the concentration of one compound and varying the concentration of other at pH 7.2. **Fig. 4.10 (A)** shows square wave voltammograms for different concentration of prednisone keeping the concentration of prednisolone constant (10 μM). The figure clearly depicts that the voltammetric peak of prednisolone remains unaltered and the peak current remained practically constant. The peak current of prednisone increased with increase in its concentration. Similarly, **Fig. 4.10 (B)** shows square wave voltammograms obtained by varying the concentration of prednisolone keeping the concentration of prednisone constant (10 μM). The prednisolone signal increases with increase in its concentration without affecting the prednisone signal, which remains almost constant. Currents observed in both the cases for the varied component were essentially same as observed during the individual determination and obeyed the relation for the calibration plot. It was found that the reduction peak of neither prednisone nor prednisolone interferes with each other in the studied concentration range and thus, the proposed method can be safely applied for their simultaneous determination.

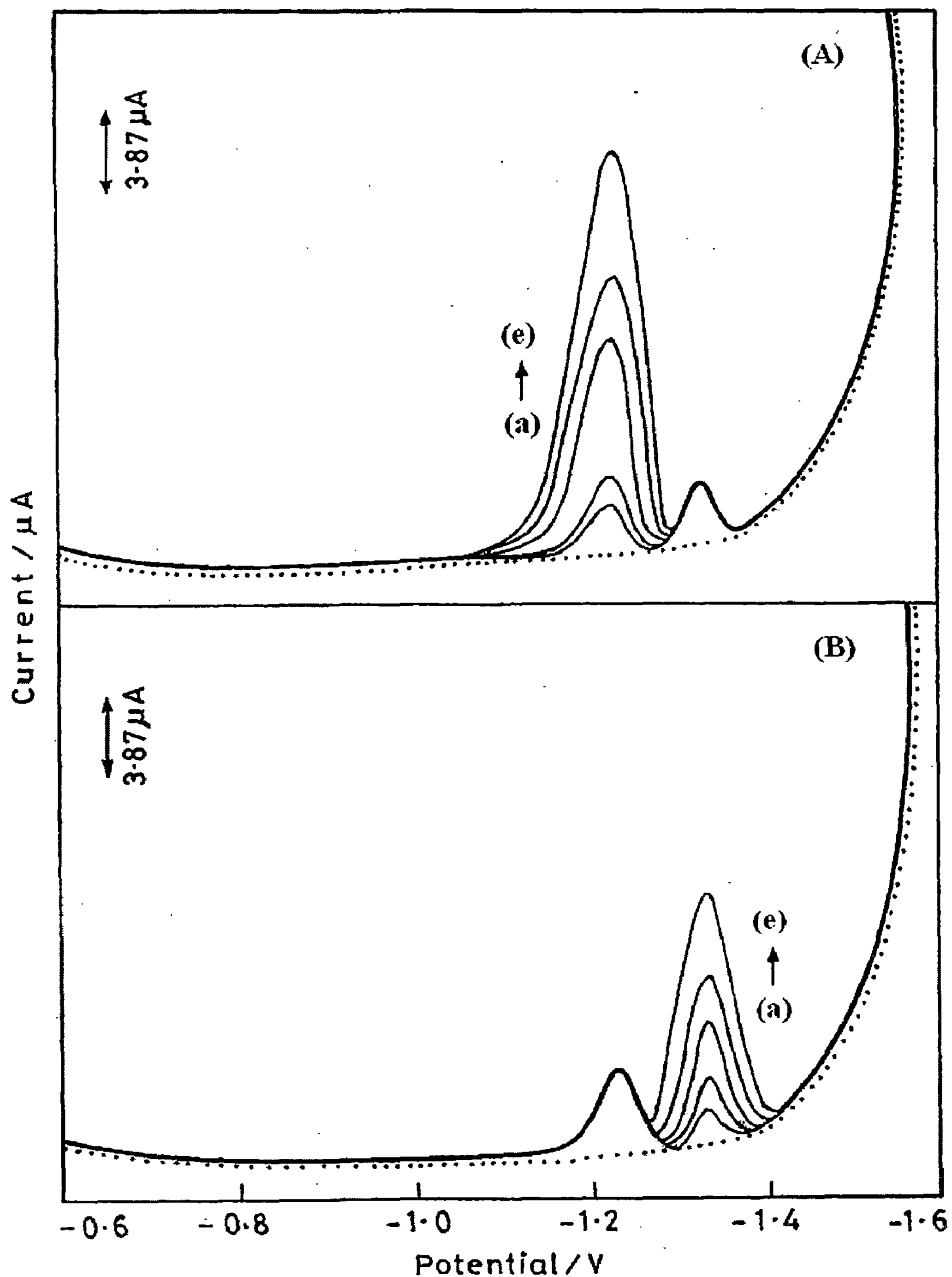


Fig. 4.10 Square wave voltammograms of blank PBS of pH 7.20 using SWNT/EPPGE (……); (A) at 10 µM fixed concentration of prednisolone and prednisone was (a) = 5, (b) = 10, (c) = 30, (d) = 40, (e) = 60 µM; (B) at 10 µM fixed concentration of prednisone and prednisolone was (a) = 5, (b) = 10, (c) = 30, (d) = 40, (e) = 60 µM.

4.3.10 Interference study

It was considered necessary to evaluate the influence of some electroactive interferents such as, uric acid, ascorbic acid, xanthine, albumin and hypoxanthine in the determination of prednisone and prednisolone, since these are common biological compounds present in noticeable amount in living systems. Study of their influence on voltammetric response of prednisone and prednisolone was carried out by recording voltammograms for mixtures containing fixed quantity (10 μM) of the analytes (prednisone or prednisolone) and varying concentration of each interferent in the range 10 – 1000 μM. The addition of these interferents did not affect the peak current of prednisone or prednisolone and no new reduction peaks were observed in the range 0 to –1.6 V. This behaviour suggested that prednisone and prednisolone can be safely determined in biological fluids. As determination is based on reduction of prednisone and prednisolone, it is understandable that common metabolites present in biological fluids do not interfere because none of them are reducible.

4.3.11 Analytical applications

4.3.11.1 Real sample analysis

To establish the utility of the developed method, prednisone and prednisolone were determined in human urine samples. In mammals including humans, interconversion of prednisolone to prednisone, has been reported, hence, residues of both steroids are found after oral administration of either of them [60-62]. Square wave voltammograms of control urine sample from healthy volunteer (a), urine sample (Sample 1) from patient undergoing treatment with prednisolone after 4 h of administration of single dose of 10 mg prednisolone tablet (Nucort Forte) (b), control urine sample spiked with 30 μM of prednisolone (c) and control urine sample spiked with 30 μM of prednisone (d) at SWNT modified edge plane pyrolytic graphite electrode are depicted in **Fig 4.11**. As can be seen, two clear peaks at – 1230 and – 1332 mV are observed in voltamograms (c) and (d), which are due to reduction of prednisone and prednisolone, respectively. Curve (a) presents votammogram of control urine and does not exhibit peaks corresponding to prednisone and prednisolone; however, peaks at same potentials in curve (b) indicate that both these steroids are excreted in urine sample (Sample 1) from the patient undergoing treatment with prednisolone. The concentrations of both the compounds in urine sample 1 were determined using developed

method and were found as 12.48 and 7.12 μM for prednisolone and prednisone, respectively.

The available sites for reduction in prednisolone are the keto groups at position 3 and 20 and in prednisone the keto groups at position 3, 11 and 20. Ketosteroid having a carbonyl group conjugated with a double bond has been reported to undergo easier reduction in comparison to isolated keto groups [90]. In prednisone reduction at carbonyl group, C-11 has been reported to be hindered due to the presence of methyl group at position 10 [66, 91]. Thus, the probable active site for reduction in both ketosteroids is carbonyl groups at position 3. Therefore, peaks at – 1230 and – 1332 mV are assigned to the reduction of carbonyl groups at position 3 to give corresponding hydroxyl groups in a $2H^+$, $2e^-$ reaction. Their interconversion in liver indicates that carbonyl group at position 11 is reduced to CHOH group [60-62] in prednisone and hydroxyl group in prednisolone is oxidized to C=O.

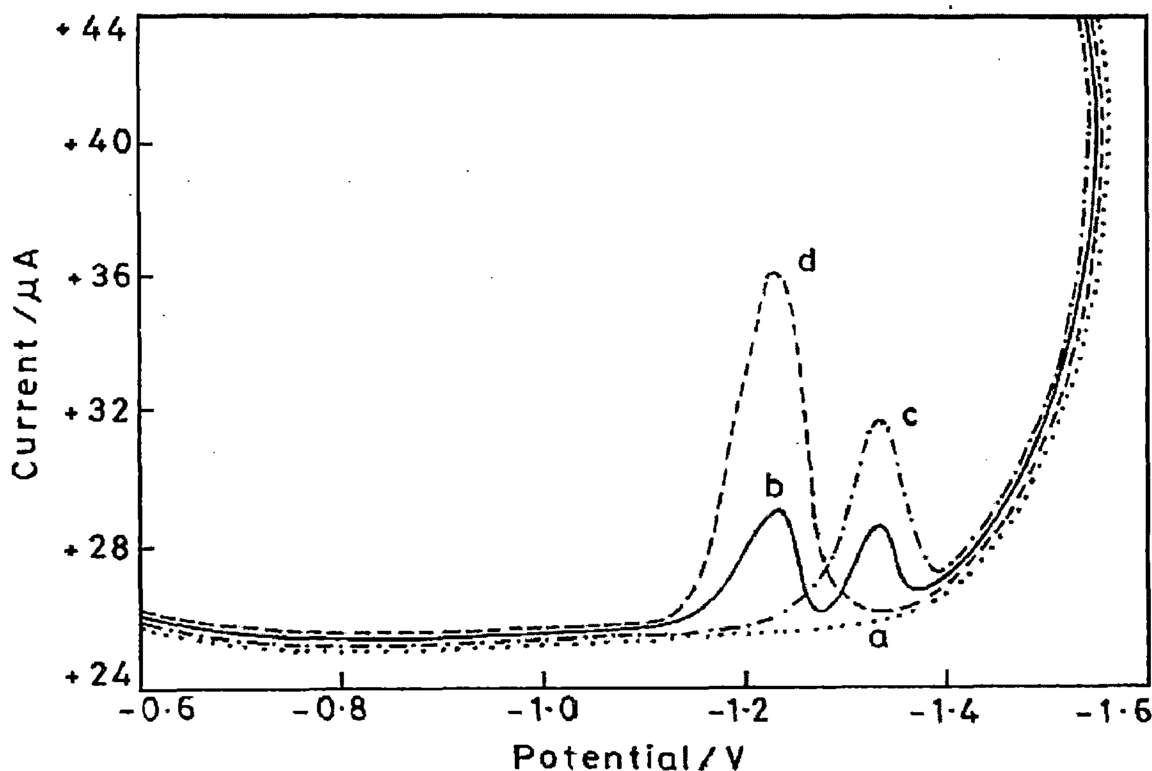


Fig. 4.11 Square wave voltammograms of control urine sample (a), urine sample of patient after administration of prednisolone tablet (b), control urine spiked with 30 μM prednisolone (c) and control urine spiked with 30 μM prednisone (d) at SWNT/EPPGE.

The concentrations of prednisone and prednisolone in human urine samples were also determined using HPLC and the results were compared with the ones observed by present method. For this purpose, various concentrations of prednisone and prednisolone standards were analyzed using HPLC and the calibration curves were obtained by plotting the peak area of the analytes peaks against concentrations. Typical HPLC chromatograms of prednisone and prednisolone is presented by curve (a) and (b) in **Fig. 4.12** and exhibit peaks at retention time ~ 7.990 and ~ 9.432 min., respectively. Urine samples were diluted 6 times prior to analysis to minimize the complexity of matrix. Curve (c) in Fig. 4.13 presents chromatogram of control urine and does not exhibit peaks corresponding to prednisone and prednisolone. The urine samples obtained from patients undergoing treatment with prednisolone after 4 h of administration of prednisolone tablet were then injected. Samples were then analyzed using HPLC method and a typical chromatogram observed for sample 1 is presented by curve (d). Two well-defined peaks at retention time ~ 7.990 and ~ 9.432 min. were noticed corresponding to prednisone and prednisolone, respectively. Other prominent peaks at R_t ~ 1.090 and ~ 1.223 min. in chromatogram were observed most probably due to the presence of major urine metabolites like uric acid and xanthine however, no attempts have been made to identify them. Finally, the concentrations of prednisolone and prednisone in human urine samples were determined using calibration curves and found as 2.00 and 1.2 μM, respectively. As urine sample was diluted six times before injection in HPLC, the concentration of prednisone and prednisolone in urine (Sample 1) was determined as 12.0 and 7.2 μM, respectively. Total three urine samples were analyzed and a comparison of prednisone and prednisolone determined by the developed method and HPLC is presented in **Table 4.3** and clearly indicates that the proposed method is in good agreement with those obtained by HPLC method.

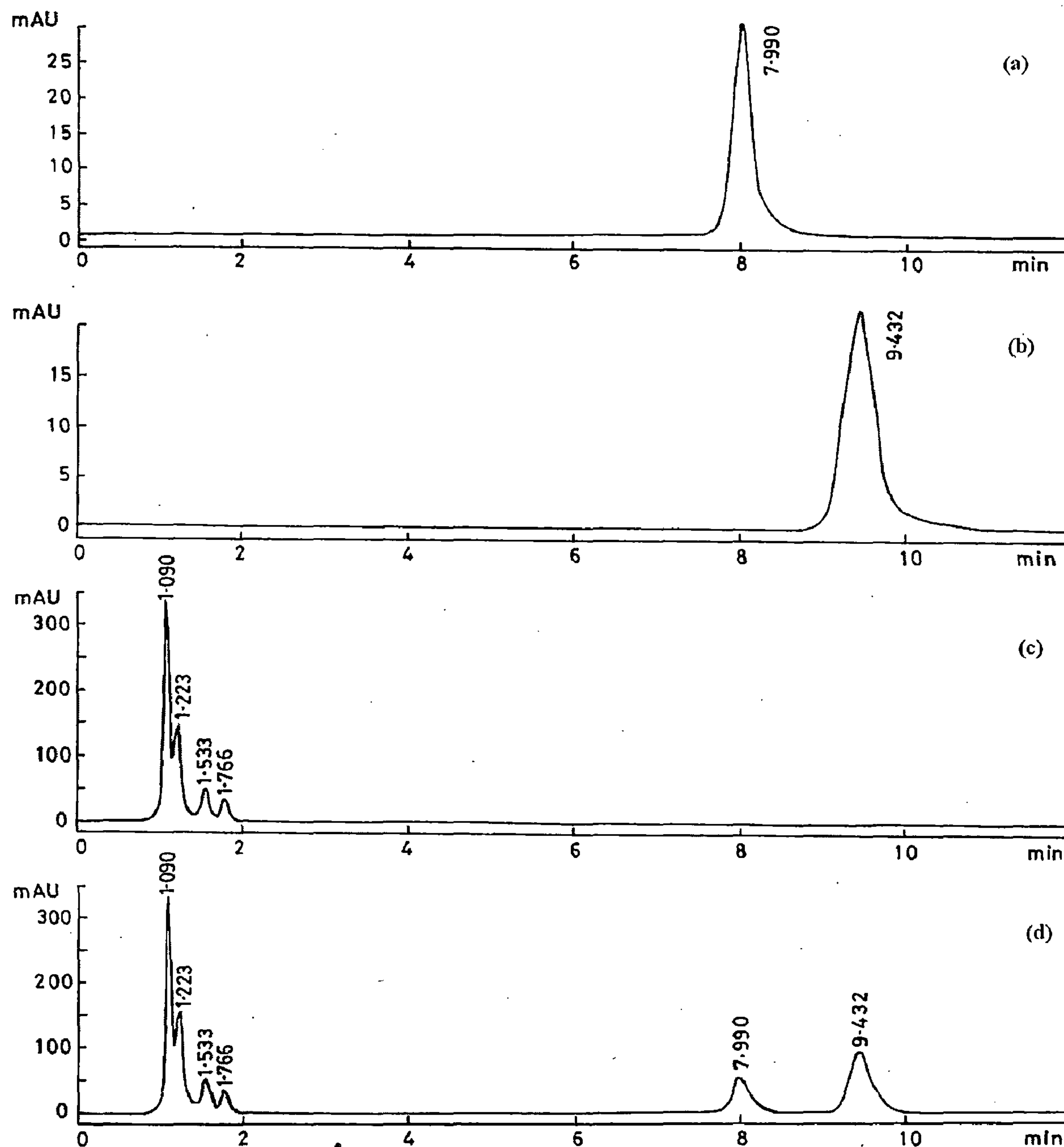


Fig. 4.12 A comparison of typical HPLC chromatograms observed for (a) standard solution of prednisone, (b) standard solution of prednisolone, (c) control urine sample, (d) urine sample obtained from patient undergoing treatment with prednisolone.

Table 4.3 Comparison of prednisone and prednisolone concentration determined by SWNT/EPPGE and HPLC in human urine after 4 h of administration of 10 mg of prednisolone tablet

Sample	Prednisolone		Prednisone	
	Modified EPPGE	HPLC	Modified EPPGE	HPLC
1	12.48	12.00	7.12	7.20
2	12.06	12.20	7.44	7.80
3	12.80	12.60	7.60	6.60

4.3.11.2 Analysis of prednisolone in pharmaceutical preparations

Prednisolone is normally prescribed for the treatment of various diseases, hence, the modified EPPGE was also used to analyze the prednisolone content in three common commercial medicinal samples viz. Nucort Forte (Unimax Laboratories, Mfg. Lic. No.; 32-B (H)), Omnacortil – 10 (Macleods Pharmaceuticals Ltd., Mfg. Lic. No.; DD/313) and Wysolone 10, (Wyeth Ltd., Mfg. Lic. No. 545). The tablets were grounded to powder, dissolved in methanol and then diluted so that the concentration of prednisolone falls in the working range. Following the proposed method the concentration of prednisolone in three commercial samples was determined using modified EPPGE. The results are summarized in **Table 4.4** and clearly indicate that the prednisolone content determined by the proposed method is in good agreement with the claimed prednisolone content in pharmaceutical preparations. It is also expected that the SWNT modified EPPGE has great potential for the determination of prednisone and prednisolone in pharmaceutical sample analysis.

Table 4.4 Determination of prednisolone in commercial tablets using SWNT modified edge plane pyrolytic graphite electrode

Sample	Stated content (mg/tablet)	Detected content (mg/tablet)	Error (%)
Nucort Forte	10	9.83	- 1.7
Wysolone	10	9.94	- 0.6
Omnacortil-10	10	9.62	- 3.8

4.3.11.3 Recovery test

Recovery experiments were also performed using standard addition method to evaluate the accuracy of the proposed method. The recovery tests of both drugs ranging from 10 μM to 50 μM were carried out utilizing SWNT modified EPPGE. Three human plasma samples obtained from healthy volunteers were spiked with known amounts of standard prednisone and prednisolone subsequently followed by recording their voltammograms. In all the cases two separate well- defined peaks were observed with E_p –1230 and – 1332 mV corresponding to prednisone and prednisolone, respectively. The concentration of the two compounds was calculated using calibration plots and the results observed are listed in **Table 4.5**. The recoveries varied in the range from 97.60 % to 101.50 % in the case of prednisolone and from 96.60 % to 103.28 % in case of prednisone. The recoveries indicate that the accuracy of the proposed voltammetric method is good.

Table 4.5 Recovery results obtained for prednisone and prednisolone in human plasma samples at SWNT/EPPGE

	Prednisolone		Prednisone	
Spiked (μM)	**Detected (μM)**	**Recovery (%)**	**Detected (μM)**	**Recovery (%)**
Sample 1				
10.0	10.15	101.50	9.88	98.80
30.0	29.84	99.47	30.24	100.80
50.0	50.60	101.20	51.64	103.28
Sample 2				
10.0	9.76	97.60	9.66	96.60
30.0	30.34	101.13	30.96	103.20
50.0	49.95	99.90	51.08	102.16
Sample 3				
10.0	10.00	100.00	9.76	97.60
30.0	30.09	100.30	30.16	100.53
50.0	49.50	99.00	50.0	100.00

4.3.12 Stability and reproducibility of SWNT/EPPGE

The stability and reproducibility of the modified electrode was evaluated by monitoring the peak current responses daily at a constant prednisolone or prednisone concentration over a period of 15 days. The experimental results indicated that the current responses showed a relative standard deviation of 3.24 % and 4.12 % for prednisone and prednisolone, respectively. After 15 days, reduction peak potentials shifted towards more negative potentials and current values were also decreased. These results suggest that the modified electrode can be safely used up to 15 days of its preparation, hence, possesses good stability.

To examine the intraday reproducibility, repetitive measurements were carried out in a solution of fixed concentration of either prednisolone or prednisone. The results of seven successive scans show a relative standard deviation of 1.06 % and 1.64 %, respectively for prednisone and prednisolone. To ascertain the reproducibility of the results further, four electrodes with almost same area were modified with the same volume of SWNT solution in DMF and their response towards the reduction of fixed concentration of prednisolone and prednisone was observed. The current obtained for the four independent electrodes showed a relative standard deviation of 2.08 % and 1.84 %, respectively confirming thereby that the results are reproducible. Thus, the modified electrode exhibits good reproducibility and stability for the determination of prednisone and prednisolone.

(C) BETAMETHASONE

4.3.13 Role of the cationic surfactant

In order to assess the effect of cetyltrimethylammonium bromide, voltammograms were recorded using four different working electrodes, viz. SWNTs-CTAB/EPPGE, SWNT/EPPGE, bare edge plane pyrolytic graphite electrode (EPPGE) and basal plane pyrolytic graphite electrode (BPPGE). Comparative study of square wave voltammograms observed for 45 nM BSP in phosphate buffer solution of pH 7.2 using above mentioned working electrodes **(Fig. 4.13)** clearly indicates that voltammetric response of BSP progressively increases on the surface of BPPGE, EPPGE, SWNT/EPPGE and SWNTs-CTAB/EPPGE, respectively. **Fig. 4.13** clearly demonstrates that at SWNTs-CTAB/EPPGE a well shaped peak at – 1062 mV is observed for the reduction of betamethasone with increased peak current however; at other electrodes comparatively poor response is

observed. The considerably improved voltammetric signals of BSP in the presence of CTAB at the electrode surface suggest that nanotubes-surfactant nanocomposite film alters the overpotential of the electrode reaction and greatly enhanced the electron-transfer conditions between BSP and electrode surface. The increase in peak current and shift of peak potential to less negative potentials can be explained by the fact that when SWNT material was dispersed in the solution of cationic surfactant CTAB rather than dimethyl formamide, positively charged CTAB adsorbed onto the electrode surface through hydrophobic interaction with strong adsorptive properties to form a compact monolayer on the electrode surface with high density of positive charges. As a consequence, negatively charged betamethasone easily reach at the electrode surface and form a complex by electrostatic interaction which facilitate the electron transfer rate and shifts the E_p to less negative potentials. Similar types of observations have been reported for surfactants as electrode surface modifiers for the determination of biomolecules [14-16].

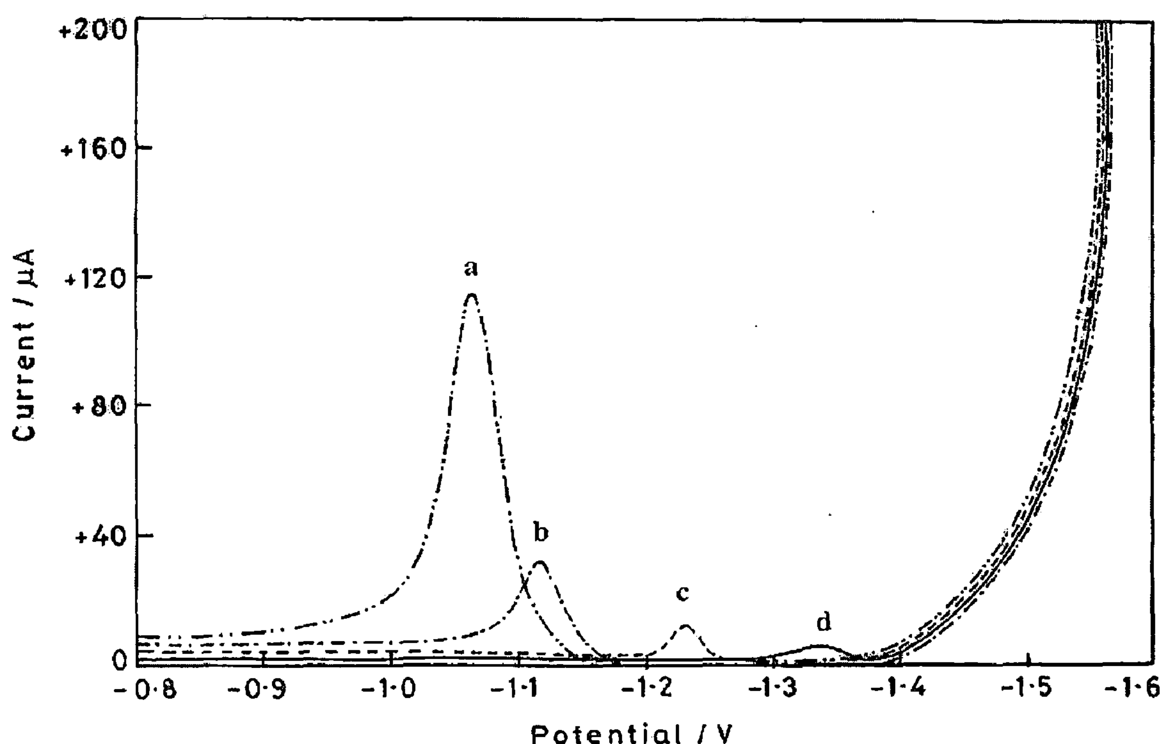


Fig. 4.13 Comparison of square-wave voltammograms of 45 nM betamethasone sodium phosphate (pH 7.2) at (a) SWNTs-CTAB film modified EPPGE (–·· –·· –··), (b) SWNT modified EPPGE (–·–·–·), (c) bare edge plane PGE (- - - -) and (d) basal plane PGE (—).

4.3.14 Voltammetric characteristics of betamethasone

4.3.14.1 Cyclic voltammetry

Encouraged from the excellent beneficial effects of cationic surfactant CTAB towards the voltammetric reduction of BSP, detailed investigations were carried out using SWNTs-CTAB nanocomposite film modified EPPGE. The electrochemical response of 15 nM betamethasone was investigated by cycle voltammetry between − 1500 to + 1500 mV in phosphate buffer solution of pH 7.2 using different pyrolytic graphite electrodes at scan rate 50 mV/s. In blank supporting electrolyte, the background current of SWNTs-CTAB/EPPGE was significantly low and no peak appeared in the whole potential region. This electrode also showed great improvements in cyclic voltammetric response of BSP in terms of large peak currents at less negative peak potential as compared to SWNT /EPPGE and bare EPPGE. **Fig. 4.14** (curve A) clearly indicates that a small bump is observed at ~ − 1332 mV using bare EPPGE and comparatively improved response is observed at SWNT/EPPGE with reduction peak at − 1148 mV (curve B of Fig. 4.15). At SWNTs-CTAB/EPPGE the peak became sharp and shifted at − 1076 mV with marked enhancement in peak current (curve C of Fig. 4.15). Cyclic voltammograms having significant increase in reduction peak current along with marked decrease in peak potential of BSP at SWNTs-CTAB/EPPGE clearly demonstrates that CTAB act as an efficient promoter to enhance the rate of electron transfer of electrode reactions. Further, only single reduction peak was observed for betamethasone at all the electrodes and absence of any peak in reverse scan suggests that BSP reduced irreversibly at bare and modified pyrolytic graphite electrodes. Cyclic voltammograms of BSP were recorded at different scan rates using SWNTs-CTAB/EPPGE. The nature of i_p / $v^{1/2}$ versus log v plot shows that the electrode reaction is controlled by adsorption [92]. 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, detailed studies were carried out using square wave voltammetry.

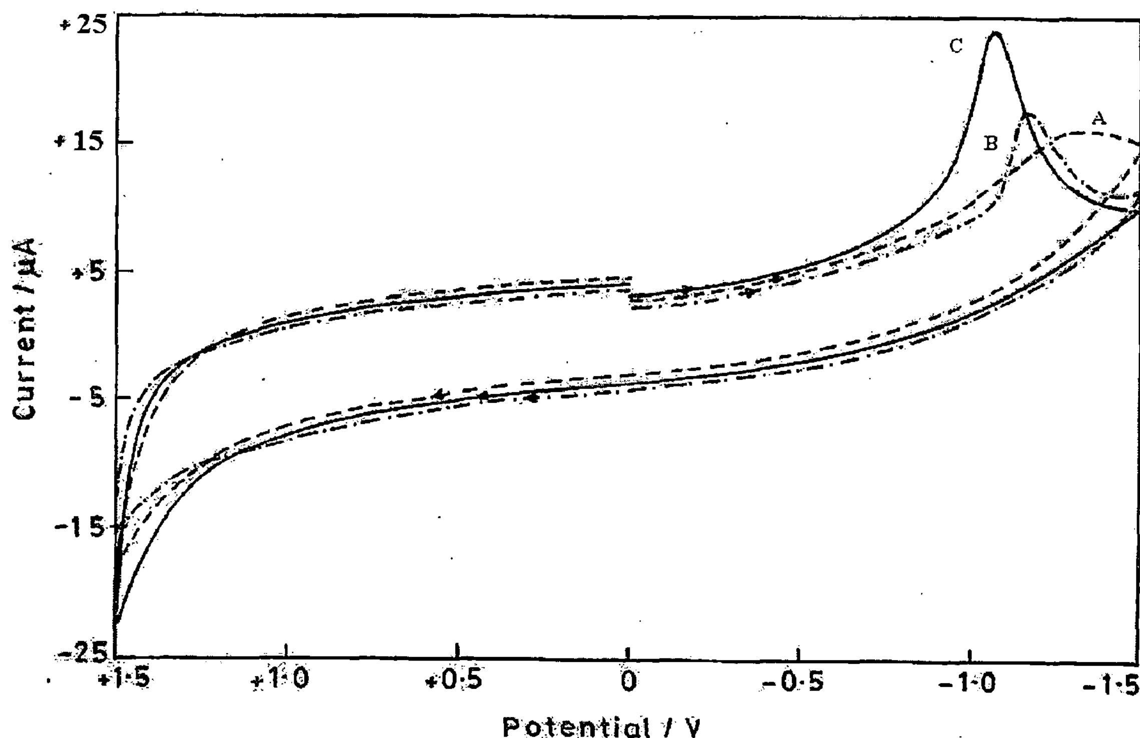


Fig. 4.14 Cyclic voltammograms of 15 nM betamethasone sodium phosphate in PBS of pH 7.2 using (a) bare EPPGE (- - - -), (b) SWNT/EPPGE (–·–·–·), (c) SWNTs-CTAB/EPPGE (—) at scan rate 50 mV/s.

4.3.14.2 pH effect

The pH of the supporting electrolyte has a noticeable effect on the electro-reduction of analyte under investigation. The electro-reduction of betamethasone was carried out by square wave voltammetry at the surface of SWNTs-CTAB nanofilm modified EPPGE over the pH range 2.4 to 10.4. The peak potential shifts to more negative values with an increase in solution pH {**Fig. 4.15 (A)**}. The linear relationship between pH value and the reduction potential ($-E_p$) can be expressed by the relation:

$$-E_p / mV = [631.4 + 58.97\ pH] \text{ versus Ag / AgCl}$$

having correlation coefficient 0.996. The slope of 59 mV/pH of E_p vs. pH relation revealed that the number of protons and number of electrons involved in the electrode reaction are same [93].

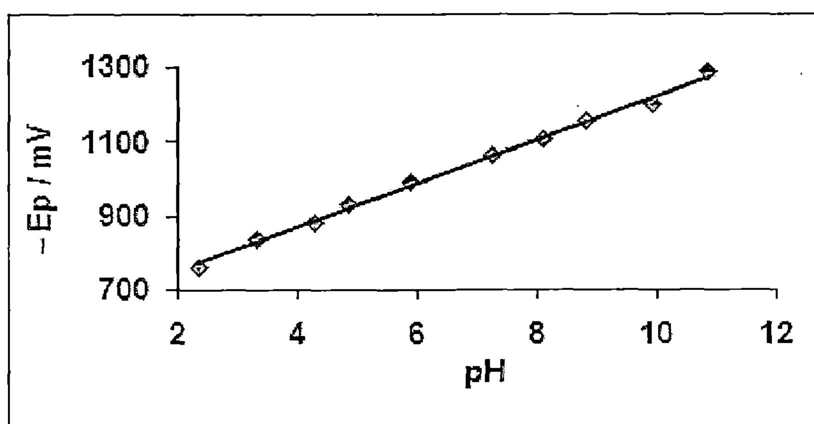


Fig. 4.15 (A) Dependence of peak potential (– E_p) on pH for 45 nM betamethasone at SWNTs-CTAB film modified EPPGE.

4.3.14.3 Effect of sweep rate

The influence of square wave frequency (*f*) on peak current and peak potential of betamethasone was examined in the frequency range 5 to 150 Hz at pH 7.2 using SWNTs-CTAB/EPPGE. A linear relationship was observed between the reduction peak current of betamethasone (45 nM) and the square wave frequency having correlation coefficient of 0.998 {**Fig. 4.15 (B)**} which further indicated adsorption of BSP at the electrode surface [83, 94]. The dependence of peak current on square wave frequency at SWNTs-CTAB film modified EPPGE can be expressed by the equation:

$$i_p / 10^{-5}\, A = 0.1756 f\ (Hz) + 9.4187$$

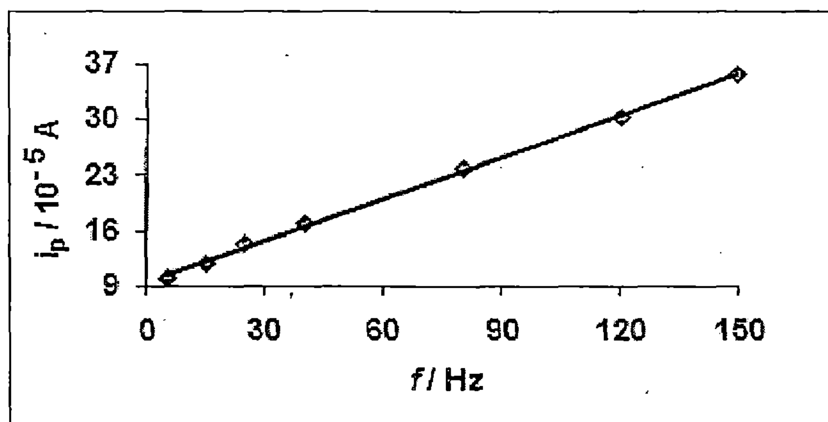


Fig. 4.15 (B) Plot of i_p versus frequency (*f*) of 45 nM betamethasone at SWNTs-CTAB nanocomposite film modified EPPGE.

It was found that the peak potential also shifted linearly towards more negative potentials over the square wave frequency range 5 to 150 Hz. The E_p versus log *f* plot was found to be linear {**Fig. 4.15 (C)**} and the variation of E_p can be expressed by the equation:

$$-E_p / \text{mV} = 180.62 \log f + 847.56$$

having correlation coefficient 0.997. These results are in agreement with the adsorption controlled irreversible electrochemical process [82, 83, 94] and also support the observations obtained from cyclic voltammetry studies.

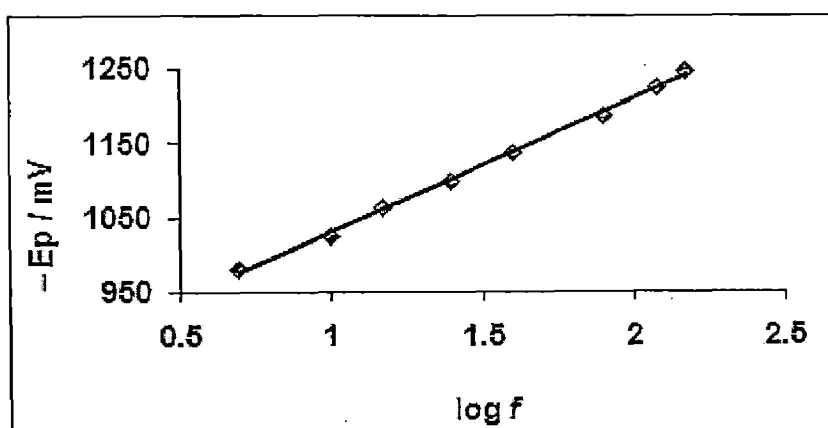


Fig. 4.15 (C) Observed dependence of peak potential (– E_p) on logarithm of square wave frequency (log *f*) for 45 nM BSP at pH 7.2 using SWNTs-CTAB/EPPGE.

4.3.14.4 Concentration study

The quantitative determination in voltammetry is based on the dependence of the peak current on the concentration of analyte under investigation. In order to determine the effect of analyte concentration on peak current square wave voltammograms of different concentration of BSP were recorded using SWNTs-CTAB/EPPGE. The reduction peak current (i_p) increased linearly as the concentration of betamethasone increased and the peak current versus concentration plot presents a good linearity in the concentration range 0.5×10^{-9} M to 100×10^{-9} M. Corresponding plot (inset) and square wave voltammograms are presented in **Fig. 4.16**.

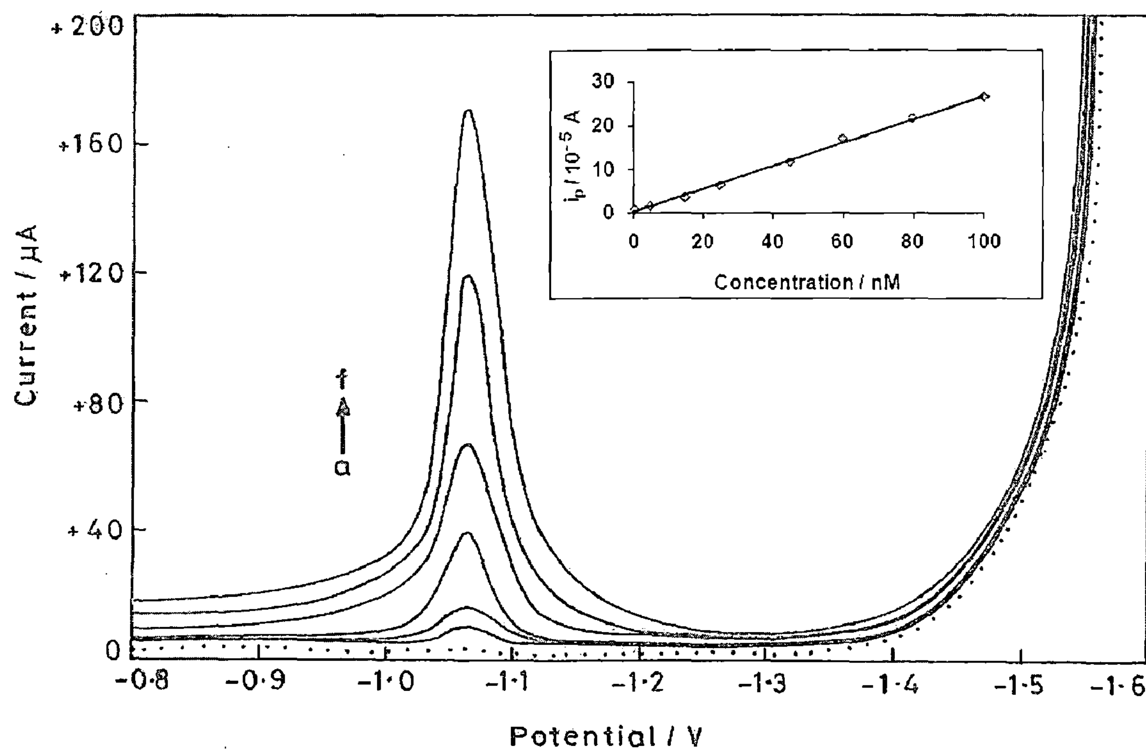


Fig. 4.16 Observed SWVs for (i) blank PBS of pH 7.20 (background) (.....) and (ii) increasing concentration of betamethasone from (a) = 0.5; (b) = 5; (c) = 15; (d) = 25; (e) = 45; (f) = 60 nM and, inset shows calibration curve for BSP using SWNTs-CTAB/EPPGE at pH 7.2.

The linear relation between i_p and concentration of BSP can be represented by the equation:

$$i_p / 10^{-5} \text{ A} = 0.267 \text{ C (nM)} + 0.0631$$

with a correlation coefficient 0.9974. The standard deviation of slope and intercept of the i_p versus concentration plot, determined from the plots of repetitive measurements was found to be 1.84% and 3.18% respectively. The detection limit of BSP was found to be 0.25×10^{-9} M at SWNTs-CTAB/EPPGE. The detection limit of BSP at SWNT modified EPPGE was also determined and found as 0.50 nM. It was observed that detection limit at SWNTs-CTAB film modified EPPGE further lowers to 0.25 nM and sensitivity increases to nearly 3.5 times [94]. The limit of quantification of the present method was found as 0.86×10^{-9} M.

4.3.15 Analytical utility

4.3.15.1 Betamethasone in urine samples

The usual doses of BSP are relatively small therefore; a highly sensitive sensor is required for the trace analysis of drug in biofluids. The SWNTs-CTAB/EPPGE has been utilized to detect BSP content in human urine samples. The urine samples were received for analysis from patients at risk for preterm delivery and receiving betamethasone. A single dose course of 0.5 mg of BSP tablet (Betnesol) daily for three consecutive days was given to all patients. Urine samples were collected after 2 h of oral administration of BSP tablet after third day. The standard addition method was adopted for the quantitative determination of betamethasone in urine samples. Successive aliquots of a standard solution were added to the solution in the cell containing the sample, and the square wave voltammogram was recorded after each addition. **Fig. 4.17** presents the square wave voltammograms observed (a) for control urine sample, (b) Control urine sample spiked with betamethasone and (c) patient urine sample after administration of betamethasone tablet. Then the actual concentration of betamethasone present in urine samples was calculated using calibration curve. **Table 4.6** indicates the results observed for BSP concentration in urine samples of pregnant women and is found to be ~ 32 nM in all the three samples. The common interfering species present in urine such as uric acid, ascorbic acid and xanthine etc. did not interfere in the determination even at 1000 times greater concentration than BSP, because these compounds are electrochemically inactive in voltammetric reduction therefore, the shape and characteristics of voltammograms observed for urine samples were the same as those obtained with the aqueous buffered solutions of pure betamethasone. The precision of the method was calculated by analyzing 10 aliquots of a sample and the relative standard deviation was less than 3.2%.

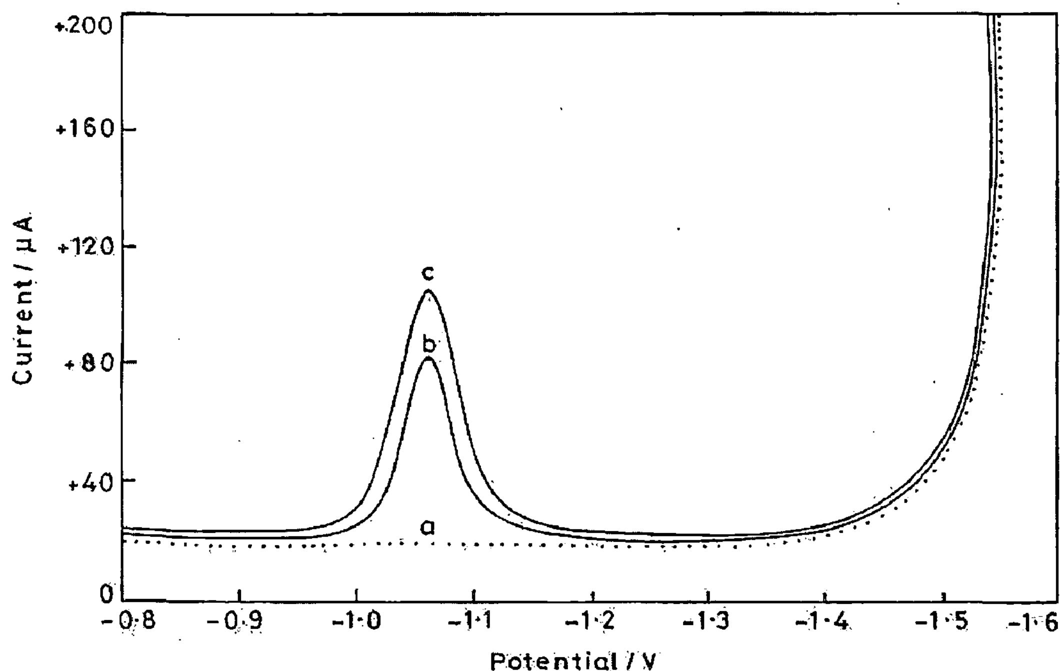


Fig. 4.17 Square wave voltammograms observed for (a) control urine sample, (b) control urine sample spiked with betamethasone and (c) patient urine sample after administration of betamethasone tablet.

Table 4.6 Observed concentration of betamethasone in urine samples of pregnant women after 2 h of betamethasone administration at SWNTs-CTAB/EPPGE

Sample	Added (nM)	Detected (nM)*	Actual Concentration (nM)
1	0.00	32.44	32.44
	0.50	32.96	32.46
	1.00	33.90	32.40
2	0.00	32.42	32.42
	0.50	33.88	32.38
	1.00	33.92	32.42
3	0.00	32.42	32.42
	0.50	32.98	32.48
	1.00	33.92	32.42

* The RSD value was < 3 % for n = 5.

4.3.15.2 Betamethasone in pharmaceutical products

The validity and sensitivity of the proposed sensor was evaluated by the determination of BSP in commercial samples to optimize the method for pharmaceutical industry. A number of pharmaceutical finished products are available in market having betamethasone as a major ingredient. Four pharmaceutical samples (tablets) containing betamethasone were analyzed using SWNTs-CTAB/EPPGE. Solutions obtained by dissolving BSP tablets are subsequently diluted so that the concentration of BSP lies in the range of the calibration plot. Then square wave voltammograms were recorded under exactly identical parameters and conditions which were employed during plotting calibration plot. Amounts of betamethasone in pharmaceutical formulations determined employing the proposed method are summarized in **Table 4.7**, and clearly indicate that the BSP content for all tablet samples detected using SWNTs-CTAB/EPPGE falls within the claimed amount. The proposed method is rapid and simple and it can be employed for the analysis of BSP in pharmaceutical preparations.

Table 4.7 Results obtained for the determination of betamethasone in pharmaceutical formulations (tablets) using SWNTs-CTAB/EPPGE

Sample compounds		Tablet label value	Experimental value*	Error (%)
		mg/tablet		
A	Stemin Forte	1.00	0.96	– 4.0
B	Betawin–S	0.50	0.48	– 4.0
C	Betnelan	0.50	0.49	– 2.0
D	Betnesol	0.50	0.475	– 5.0

* RSD value was $< 2.6\%$ for $n = 3$.

4.3.16 Stability and reproducibility of SWNTs-CTAB/EPPGE

As the modified electrode can be prepared quickly hence, the long term stability of the electrode is not of prime importance. However, the stability of SWNTs-CTAB/EPPGE was evaluated by measuring the reduction peak current for fresh solutions of BSP over a period of 15 days. Experimental measurements indicate that only ± 6.8 % discrepancy in

current response was observed for 15 days with the fresh solutions prepared daily compared to the original reduction current values. Thereafter, a noticeable decrease in peak current was observed and peak potential also shifted to more negative values, thus it is recommended that modified electrode should not be used after 15 days.

The reproducibility of the modified electrode was evaluated by successive measurements ($n = 6$) of 5.0×10^{-6} mol L^{-1} betamethasone solution. The corresponding relative standard deviation of 3.8 % confirms that the results are reproducible. The reliability of the fabrication procedure was also examined by measuring voltammetric response of fixed concentration of betamethasone solution under identical conditions using four different electrodes of same area, which were fabricated independently by the same procedure. The corresponding relative standard deviation of ± 4.2 % indicates that the modification procedure is quite reliable.

4.4 CONCLUSIONS

The nanotubes modified sensors developed for the determination of amlodipine, prednisone, prednisolone and betamethasone offer marked decrement in peak potential, high sensitivity, low detection limit and stable voltammetric sensing. As these drugs are extensively abused by athletes for doping therefore, it is expected that the determination of these steroids in human body fluids would provide a simple and fast method for detecting doping cases at the site of competitive games. A detailed comparison of MWNT and SWNT modified EPPGEs towards the oxidation of amlodipine indicates the enhanced electrocatalytic performance of SWNTs. It was also found that surface modification of EPPGE with SWNTs-CTAB nanocomposite film remarkably enhances the electrochemical response of betamethasone as compared to SWNTs-DMF film. The method using CTAB as electrode surface modifier has also been found to produce a four fold increase in surface area of the edge plane pyrolytic graphite electrode, which is one of the potential regions for electrocatalytic properties of modified electrodes. The developed methods have been satisfactorily applied for determination of all the drugs in pharmaceutical formulations as well as human body fluids. The simplicity, sensitivity, low detection limit and short analysis time of the proposed procedure makes it useful for the fast analysis of drugs/doping agents in pharmaceutical formulations and human body fluids.

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Chapter 5

DETERMINATION OF DRUGS AND BIOMOLECULES AT C_{60} MODIFIED ELECTRODES

5.1 INTRODUCTION

The electrocatalytic activity of carbon nanotubes is assigned in recent years due to the presence of metallic impurities [1, 2]. It has been reported that some metallic impurities are encapsulated in carbon nanotubes, which are inaccessible to fluids, and thus unavailable for most biological reactions. Thus, the metal which is completely encapsulated in impermeable carbon shells and not in contact with fluid does not play any role [3]. The "fluid accessible metal" is probably an important property of carbon nanotubes (rather than total metal present) and is believed to be responsible for electrocatalytic activity of CNTs [4]. It is difficult to remove all the metallic content without damaging the structure and so the acid treatment (12 h and 72 h) is not influential for the complete removal of embedded metallic impurities [1, 4, 5]. The electrocatalytic nature of fullerene has also been a topic of interest in the recent years. It has been reported that the graphite impurities present in fullerene are responsible for the observed electrocatalysis [6]. On the other hand, the surface oxygenated species are claimed to promote electro catalysis rather than the fullerene [7]. Partially reduced conductive K_3C_{60} film formed at the surface of electrode has also been assigned special electrocatalytic properties [8, 9]. The aim of the present work is to resolve the conflicting views and find out the actual reason of electrocatalytic property of fullerene. Hence, it is considered enviable to investigate the effect of embedded metallic impurities (Fe, Cu, Co, Ni) of fullerene, which are accessible to fluids on which fullerene is casted. In this chapter, the effect of embedded metallic impurities of fullerene, the effect of substrate and the application of fullerene-C_{60}-modified electrodes for determination of nandrolone and simultaneous determination of adenine and 2'-deoxyadenosine (2'-dAdo) have been discussed.

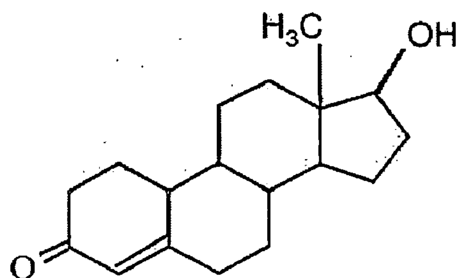
The abuse of anabolic androgenic steroids (AASs) at supratherapeutic doses is a problem not only in the world of sports, but also among non athletes using AASs to improve physical appearance and to become more bold and courageous [10, 11]. Nandrolone (17 β – hydroxyestra – 4 – en – 3 – one) is an anabolic androgenic steroid [12] banned in sports by the International Olympic Committee and World Anti-Doping Agency as it is extensively used by bodybuilders and athletes for the purpose of enhancing athletic performance [13-17]. The International Olympic Committee has set a limit of 2.0 ng per mL of urine as the upper limit, beyond which an athlete is suspected of doping [18]. On July 14, 2008 a Pakistani cricketer Mohammad Asif has been tested positive for nandrolone during

Indian Premier League [19]. Two well known ester derivatives of nandrolone are nandrolone decanoate and nandrolone phenylpropionate out of which nandrolone decanoate is the most commonly used. The most widely used pharmaceutical formulation of nandrolone decanoate is Deca – Durabolin which is administered by intramuscular injection in an oily base. The positive effects of the drug include muscle growth, appetite stimulation, increased red blood cell production and bone density [20-22]. Clinically, it is used in treating anemia, neoplasia including breast cancer, rebuilding of muscles after debilitating disease and treatment of osteoporosis in postmenopausal women [23, 24]. Nandrolone decanoate prevents osteopenia and inhibits bone turnover in monkeys [25]. In view of the clinical importance and increased abuse of nandrolone by athletes, it is worthy to analyse its concentration in body fluids as well as in pharmaceutical formulations. Square wave voltammetry is the most sensitive pulse technique and it effectively suppresses background current that makes it versatile for analytical purposes. The present studies report effect on oxidation peak current and potential of nandrolone as a function of relative removal of the embedded metal impurities of fullerene. In the present investigation, a comparison of EPPGE as a substrate for fullerene modification has also been made with other conventional electrodes like indium tin oxide (ITO), glassy carbon electrode (GCE), gold and basal plane pyrolytic graphite electrode (BPPGE). The developed protocol has also been utilized for the determination of nandrolone in pharmaceutical formulations.

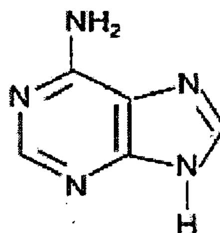
Adenine is one of the two purine nucleobases and 2'-dAdo is one of the purine 2'-deoxyribonucleosides present in deoxyribonucleic acid (DNA), and therefore, both are essential molecules of life and evolution. Adenine is of tremendous biological significance, since it is one of the nitrogenous bases, found in deoxyribonucleic acid and ribonucleic acid to make up genetic information. It is a component of adenosine triphosphate 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 plays an important role in protein synthesis. 2'-dAdo is a carbohydrate derivative of adenine, in which adenine is linked through its N-9 position via a N-glycosidic β-D erythro- pentofuranose β bond to 2'-deoxy –D-ribose [26]. 2'-dAdo has been reported to be a powerful stimulator of human sperm motility [27]. It selectively kills nonneuronal cells without affecting neuronal cells, and without any adverse effect on the viability of parasympathetic neurons [28]. It is an important precursor of some antitumor and antileukemic drugs synthesis [29, 30] and also exhibits a cardio protective action [31]. Simultaneous determination of adenine and 2'-dAdo has great significance to bioscience

and clinical diagnosis since both these compounds occur simultaneously in body fluids. Intracellular levels of adenine and 2'-dAdo are important, because their altered concentration cause various metabolic disorders in human system [32-35]. Endogenously produced adenine is mainly derived from the cleavage of 5'-methylthioadenosine [36]. The conversion of 2'-dAdo to adenine represent a protective device to control the plasma level of 2'-dAdo when the activity of adenosine deaminase (ADA) is inhibited [37]. Therefore, the simultaneous determination of 2'-dAdo and adenine is the subject of considerable interest, especially in human body fluids particularly urine in case of carcinoma. Since, in the case of hepatocellular carcinoma, the level of adenine has been found to increase considerably [38].

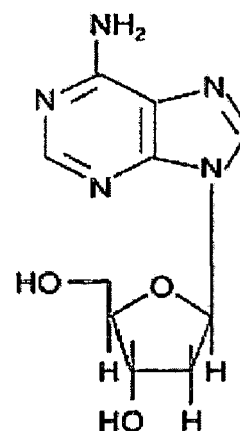
Many chromatographic and electrochemical methods have been developed for detection and quantification of both compounds individually or with other compounds [30, 39-41]. However, to the best of our knowledge, no report is found for simultaneous determination of these two compounds in biological fluids by voltammetry. The present study deals with the simultaneous determination of adenine and 2'-dAdo employing square wave voltammetry at a fullerene – C_{60} – modified glassy carbon electrode (C_{60}/GCE). Due to its catalytic activity fullerene – C_{60} – modified GCE shifts the oxidation potential towards less positive direction with the simultaneous enhancement of peak current for both the analytes. Thus, improves the sensitivity and detection limit of electrochemical determinations. The proposed sensor has been utilized for the detection of adenine and 2'-dAdo in urine samples of leukemic patients and the results were also cross validated using high performance liquid chromatography.



Nandrolone



Adenine



2'-Deoxyadenosine

5.2 EXPERIMENTAL

5.2.1 Instrumentation

Voltammetric experiments were performed using BAS (Bioanalytical Systems, West Lafayette, USA) CV-50W voltammetric analyzer which has a three electrodes single compartment cell system. A platinum wire as an auxillary electrode, Ag/AgCl (3 M NaCl) as reference electrode (BAS Model MF-2052 RB-5B) and fullerene – C_{60} – modified EPPGE and GCE as working electrodes were used for voltammetric determinations. The edge plane pyrolytic graphite was obtained from Pfizer Inc., New York, USA. The pH of the buffers was measured using a Century India Ltd. Digital pH meter (Model CP-901) after proper standardization. The determination of metallic content of fullerene was carried out using Perkin Elmer Sciex ELAN DRC-e ICP-MS. All experiments were carried out at an ambient temperature of 25 ± 2 °C referred to Ag/AgCl electrode. HPLC studies were performed on Agilent 1100 series system attached with reverse phase C_{18} column.

5.2.2 Reagents

Nandrolone decanoate injection was obtained from Ind-Swift Ltd., Parwanoo, H.P. Buckminsterfullerene (C_{60}) was purchased from Aldrich, USA of purity 98%. Phosphate buffer solutions were prepared according to the method of Christian and Purdy [42]. Adenine was purchased from Koch Light, U.K and 2'-dAdo was obtained from Fluka chemie GmbH, Switzerland and both were used as received. Uric acid, ascorbic acid, hypoxanthine, xanthine and albumin from bovine serum were obtained from Merck and used as received. All the chemicals and reagents were of analytical grade and were used as received without any further purification. Double distilled water was used for all experiments.

5.2.3 Procedure

Stock solution (1 mM) of nandrolone decanoate was prepared by diluting the injection content (25 mg in 1 mL) with double distilled water and then stored at low temperature. Stock solution of adenine and 2'-dAdo (2 mM) were also prepared in double distilled water. Required amount of the stock solution was added to 3 mL of phosphate buffer solution (1.0 M, pH 7.2) and the total volume was made to 6.0 mL with double distilled water. Optimized parameters for nandrolone SWV study were: initial E: 0 mV, final E: 1200 mV, square wave amplitude (E_{sw}): 50 mV, potential Step (E): 4 mV, square wave frequency (f): 15 Hz. The optimized experimental conditions for adenine and 2'-dAdo

SWV study were: square wave amplitude, 25 mV; square wave frequency, 20 Hz; sensitivity, 100 μA/V, step height, 4 mV. For HPLC experiments the mobile phase used was a mixture of NaH_2PO_4 (0.01M): methanol (60:40) at a flow rate of 0.5 ml/min. The absorbance of the eluent was recorded at 254 nm.

5.2.4 Preparation of fullerene-C_{60}-modified electrodes

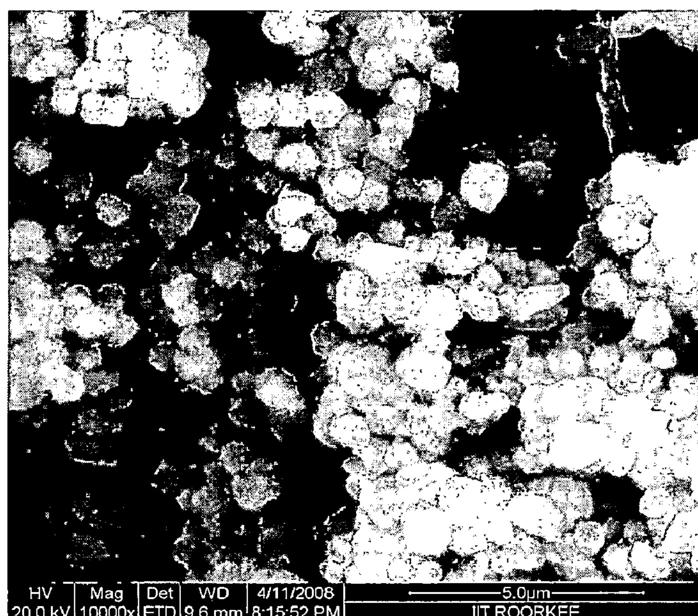
The surface of GCE and gold electrode was cleaned to a mirror like surface finish by polishing with alumina using micro cloth pads (BAS, USA) followed by rinsing with distilled water and dried. Electrodes were then sonicated in 0.2 M H_3PO_4 solution, to remove adhered alumina and then rinsed thoroughly with distilled water and dried. The bare EPPGE and BPPGE were rubbed on the emery paper and then washed with double distilled water and dried. A sheet of ITO was washed with sonication in acetone, ethanol and distilled water. All the electrodes were then ready for modification.

Stock solution of fullerene (98 %) was prepared by dissolving it in dichloromethane (150 μM) using ultrasonic bath. Fullerene solution (40 μL) was coated onto the surface of each electrode using microsyringe followed by drying of the electrodes in a stream of hot air. The C_{60} film formed on the surface of the electrodes was partially reduced in 1 M KOH in the potential range 0.0 to – 1.5 V at 10 mVs^{-1} [43]. Such a treatment made fullerene-C_{60} film sufficiently conducting due to the formation of K_3C_{60} [44]. The electrodes surface were then equilibrated in phosphate buffer solution (PBS) (50 mM) of pH 7.2 by cyclic scanning in the potential range of 550 to – 50 mV at a scan rate of 20 mVs^{-1} for 20 min under nitrogen atmosphere. The untreated fullerene modified electrodes were then ready for use.

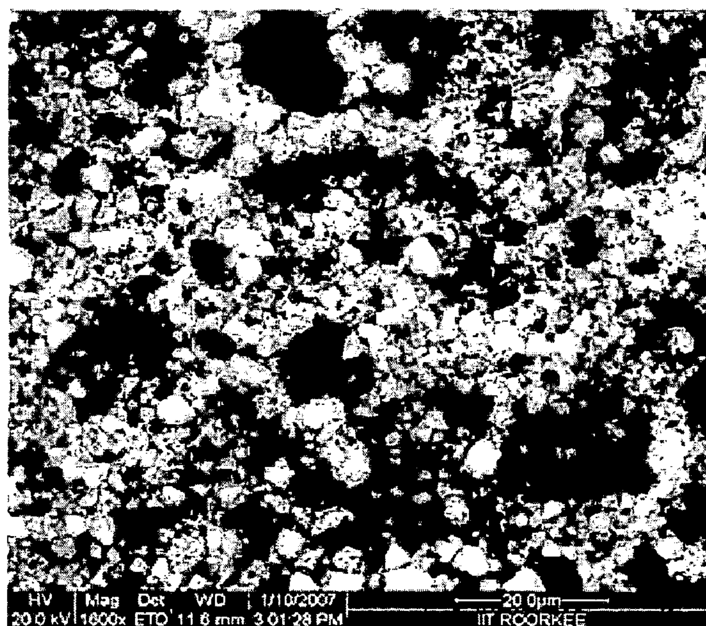
For the preparation of purified (12 h treated) and super-purified (72 h treated) fullerene, the 98 % pure fullerene was acid treated. Treatment with nitric acid for 12 h resulted in purified fullerene and treatment with nitric acid for 12 h followed by hydrochloric acid for 60 h yielded super-purified fullerene. The acid treated fullerenes were then washed with double distilled water several times and kept in air for drying. They were then ready to be used for modification.

The effect of amount of C_{60} casted on electrodes surface was also studied by taking different amount of stock solution. It was found that with increase in amount upto 40 μL the peak current of adenine increased and becomes constant at higher concentration of C_{60}. Thus, 40 μL of the stock solution was considered as optimum and used in subsequent studies. The surface morphology of untreated fullerene modified EPPGE and GCE is shown

in **Fig. 5.1** which clearly indicates deposition of C_{60} particles. The electrodes surface was cleaned after each run by applying a potential of – 200 mV for 60 s to desorb any adsorbed material.



(A)



(B)

Fig 5.1 A typical FE-SEM images of (A) fullerene – C_{60} – modified EPPGE and (B) fullerene – C_{60} – modified GCE after partial reduction in KOH.

5.2.5 Biological samples

Two urine samples were collected from laboratory personnel. The collected samples were diluted 10 times with 0.1 M phosphate buffer solution (pH 7.2) to minimize matrix complexity. The diluted urine samples were then spiked with different amounts of adenine and 2'-dAdo and voltammograms were recorded. Similarly blood samples from three healthy volunteers were obtained from the Hospital of IIT Roorkee. The blood with ethylenediaminetetraacetic acid (EDTA) as anticoagulant was ultra-centrifuged and the supernatant blood plasma was obtained. The collected plasma samples were diluted 10 times with 0.1 M phosphate buffer solution (pH 7.2). The diluted blood samples were then spiked with adenine and 2'-dAdo solutions and voltammograms were recorded again. As it has been reported that amount of adenine present in the urine of leukemic patients is much higher than normal persons, hence, the practical utility of the modified electrode is also examined by analyzing urine sample of two second last stage hepatocellular carcinoma patients (Anthropometric data–Female; age 49 and 54 years; height 152 and 160 cms; weight 69 and 75 kg) undergoing treatment at the Institute Hospital after 10 times dilution.

5.3 RESULTS AND DISCUSSION

(A) NANDROLONE

5.3.1 Effect of substrate

To study the effect of the substrate on the voltammetric behavior of nandrolone, five different working electrodes were used which included fullerene modified BPPGE, gold, GCE, ITO and EPPGE. The exposed are of the electrodes to the solution was nearly 2 mm^2 in all the cases. A comparison of the square wave voltammograms (SWVs) recorded for 5 nM nandrolone in phosphate buffer solution at pH 7.2 is presented in **Fig. 5.2.** In the case of fullerene modified BPPGE, peak is noticed at ~ 698 mV and at fullerene modified gold it is observed at ~ 642 mV. GCE and ITO serve as better substrates for fullerene modification in comparison to BPPGE and gold giving peaks at ~ 630 and ~ 591 mV with an increase in peak current. It is evident from the figure that the oxidation of nandrolone occurs at much lower potential (~ 456 mV) at fullerene modified EPPGE with a marked enhancement in the current response. Thus, EPPGE acts as the best substrate in comparison to the other four electrodes for modification by fullerene. EPPGE have a strong tendency to adsorb compounds and possesses a large potential window. Hence, EPPGE was chosen as the substrate for fullerene modification in further studies.

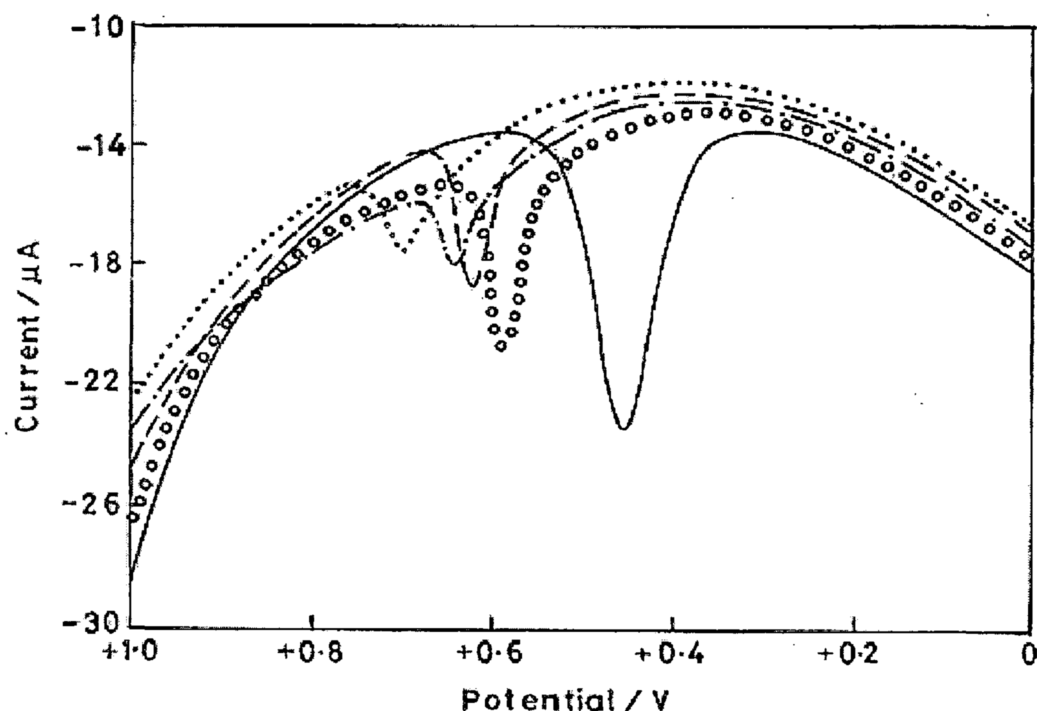


Fig. 5.2 A comparison of square-wave voltammograms of 5 nM nandrolone at pH 7.2 at untreated fullerene – C_{60} – modified EPPGE (—), ITO (o o o), GCE (- - - -), gold (–·–·–·) and BPPGE (······).

5.3.2 Role of metallic impurities

The electrocatalytic nature of carbon nanotubes was assigned due to the presence of metallic impurities in it and this prompted us to investigate the function of embedded metallic impurities of fullerene. For this purpose, we used acid treated (12 h and 72 h) and untreated 98 % fullerene. **Fig. 5.3** shows a comparison of the voltammetric response of nandrolone on bare EPPGE, super-purified fullerene modified EPPGE, purified fullerene modified EPPGE and untreated fullerene modified EPPGE. It is observed that bare EPPGE gave a poor response with a small peak at ~ 653 mV. A significant increase in the peak current with lowering of peak potential was observed at untreated fullerene modified EPPGE in comparison to purified and super-purified fullerene modified EPPGE. This clearly indicates that the embedded metallic impurities present in fullerene play a crucial role in its electrocatalytic properties. Metallic contents of the different samples were then determined using Inductively Coupled Plasma- Mass Spectrometry (ICP-MS). It can be seen in **Table 5.1** that with the decrease of metallic content in fullerene there is a sharp decrease in peak current along with an increase in the peak potential of nandrolone. Thus, it

is suggested that 98 % pure fullerene should be used directly for electrochemical experiments without any complex and time consuming purification procedure. All studies described further in this paper were carried out using untreated fullerene modified EPPGE.

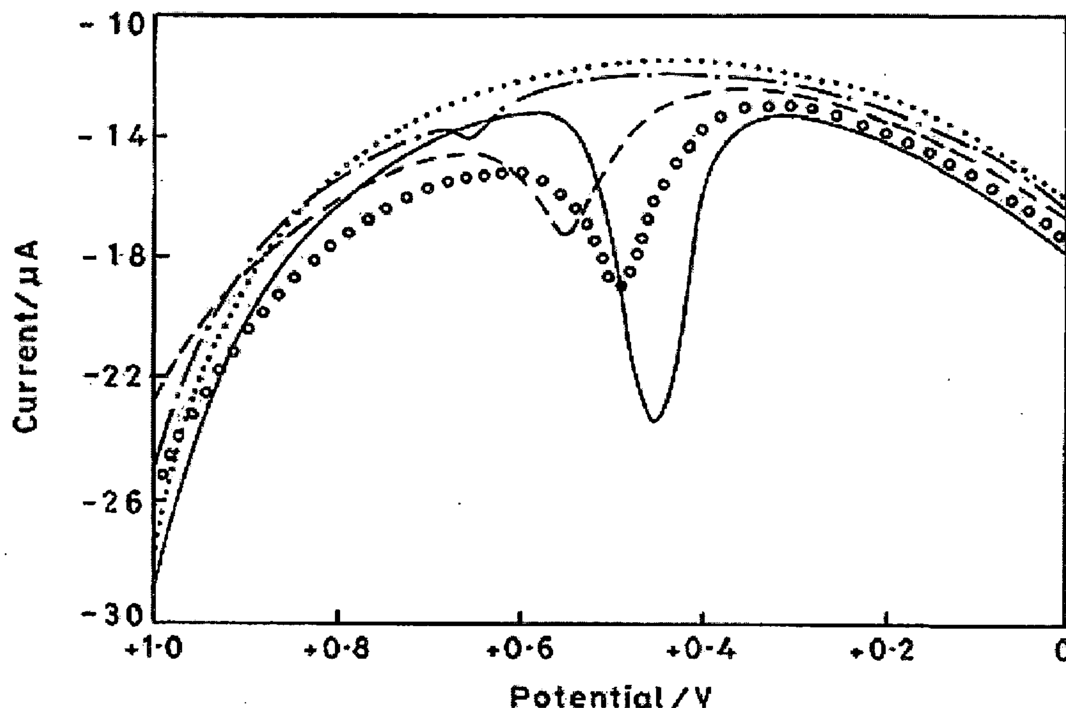


Fig. 5.3 Square wave voltammograms recorded for 5 nM nandrolone at pH 7.2 at untreated (—), purified (o o o) and super purified (- - - -) fullerene – C_{60} – modified EPPGE, bare EPPGE (—·—·—) and background phosphate buffer solution (pH 7.2) at untreated fullerene – C_{60} – modified EPPGE (······).

Table 5.1 Effect of metallic contents of fullerene on peak potential and peak current of nandrolone in phosphate buffer of pH 7.2

Sample	Metallic content in (%)				Nandrolone	
	Fe	Cu	Co	Ni	E_p (mV)	i_p (μA)
Untreated fullerene	0.416	0.191	0.007	0.392	456	11.7
Purified fullerene	0.162	0.164	0.001	0.263	492	7.25
Super-purified fullerene	0.099	0.071	0.001	0.086	548	5.43

5.3.3 Influence of pH

The pH of the supporting electrolyte has a significant effect on the oxidation peak of nandrolone at untreated fullerene modified EPPGE. Effect of pH was studied in the range of 2.4 to 10.9 at a square wave frequency of 15 Hz. The potential of the oxidation peak of nandrolone shifted to less positive potentials with increase in pH as seen in **Fig. 5.4**. The linear dependence of the peak potential on pH at the modified electrode is represented by the following equation:

E_p (pH 2.4 – 10.9) = [788 – 48.61 pH] mV versus Ag/AgCl $(R^2 = 0.9962)$

The dE_p/dpH value of ~ 49 mV/pH indicates that equal number of protons and electrons are involved in the oxidation of nandrolone. The hydroxyl group present at the 17th position in nandrolone undergoes oxidation by a loss of $2e^-$, $2H^+$ to give a cyclic keto group [45].

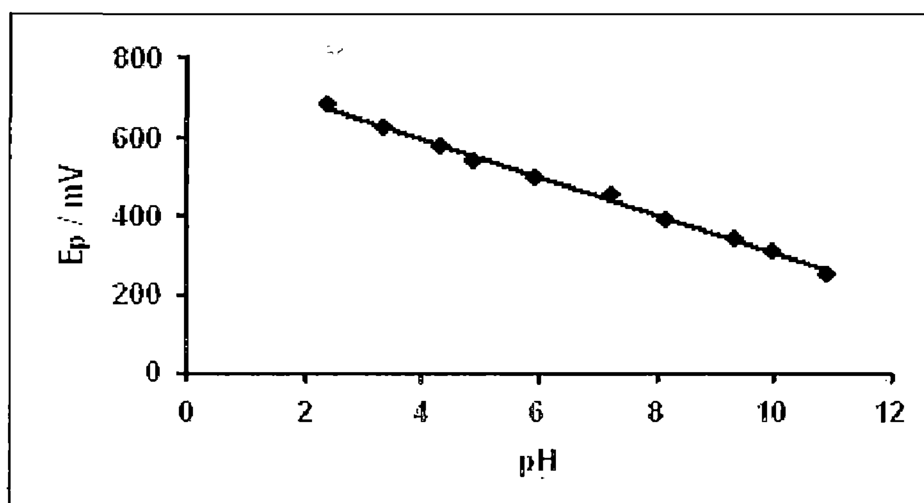


Fig. 5.4 Observed dependence of peak potential (E_p) on pH for 5 nM nandrolone at untreated fullerene – C_{60} – modified EPPGE.

5.3.4 Calibration curve

The effect of concentration of nandrolone on peak current was studied at optimized parameters in the concentration range 0.01 – 50 nM. The current values are reported as an average of at least three replicate determinations and are obtained by subtracting the background current. The peak current increased with increase in concentration and the

calibration curve was found to be linear (**Fig. 5.5**). The linearity of peak current versus concentration can be given by the following regression equation:

$$i_p\ (\mu A) = 1.838\ C\ (nM) + 0.1934 \qquad (R^2 = 0.9967)$$

where C is the concentration of nandrolone. The sensitivity of the proposed method is 1.838 $\mu A\ nM^{-1}$ and the detection limit was found to be 1.5×10^{-11} M.

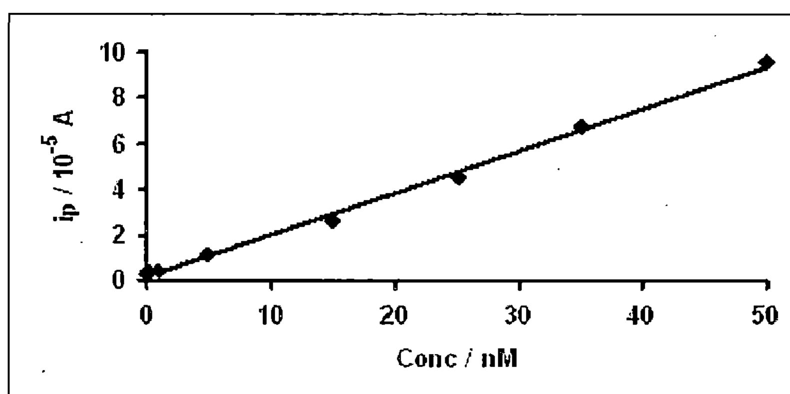


Fig. 5.5 Calibration plot observed for nandrolone at untreated fullerene – C_{60} – modified EPPGE at pH 7.2.

5.3.5 Effect of square wave frequency

The dependence of peak current (i_p) and peak potential (E_p) of nandrolone on the square wave frequency (*f*) was studied in the range 5 – 150 Hz. The peak current was found to increase linearly with square wave frequency and the relation between i_p and f can be represented by the following equation:

$$i_p = 0.0954 f + 0.2943 \qquad (R^2 = 0.9942)$$

This type of voltammetric response indicates that the nature of electrode reaction is adsorption controlled [46-48]. The peak potential of nandrolone shifted towards more positive potential with increase in square wave frequency. The plot of E_p versus $\log f$ was linear (**Fig. 5.6**) and the variation can be expressed by the equation:

$$E_p\ (mV) = 117.74 \log f + 313.91 \qquad (R^2 = 0.9925)$$

Such behaviour indicated the nature of redox reaction as reversible [48].

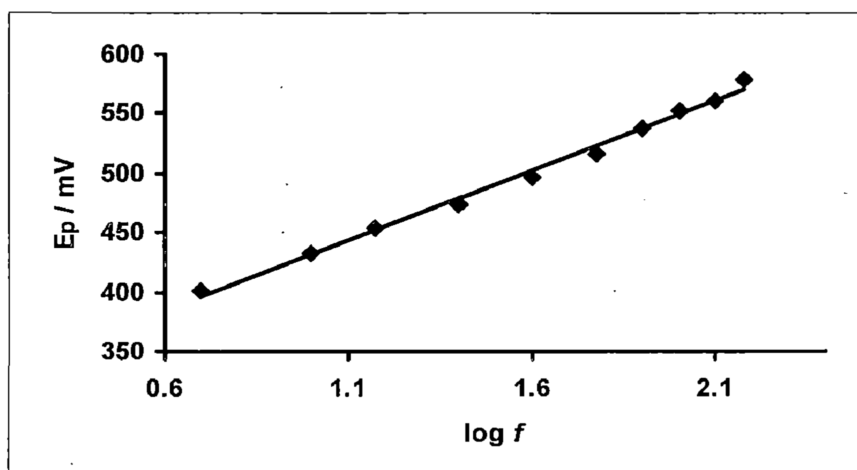


Fig. 5.6 Dependence of peak potential (E_p) on logarithm of square wave frequency for 5 nM nandrolone at untreated fullerene – C_{60} – modified EPPGE at pH 7.2.

5.3.6 Determination of nandrolone in pharmaceutical formulations

The practical utility of the proposed method was examined by analyzing three commercial medicinal samples containing nandrolone viz. Deca Neurophen-25 (Ind-Swift Ltd., Parwanoo, H.P), Seadec-25 (Growell Pharmaceuticals, Bahadurgarh) and Durabolin-25 (Organon India Ltd., Kolkata). The content of the injection was diluted to get the concentration of nandrolone in the working range and then square wave voltammograms were recorded using untreated fullerene modified EPPGE. The concentration of nandrolone in the three pharmaceutical formulations was determined with the help of calibration curve. **Table 5.2** shows that the content values determined by the proposed method for all the commercial samples are very close to the claimed amount. Thus, the analysis of nandrolone in pharmaceutical samples confirms the applicability of the developed method.

Table 5.2 Comparison of observed and reported nandrolone concentration in pharmaceutical formulations at untreated fullerene – C_{60} – modified EPPGE

Sample	Reported concentration (mM)	Observed concentration (mM)	Error (%)
Deca Neurophen	58.32	56.85	- 2.5
Durabolin	61.49	60.81	- 1.1
Seadec	58.32	56.04	- 3.9

(B) ADENINE AND 2'DEOXYADENOSINE

5.3.7 Comparison of bare and modified electrodes

The square wave voltammograms (SWVs) recorded for a binary mixture of 50 μM adenine and 50 μM 2'-dAdo at bare and fullerene – C_{60} – modified GCE in 0.1 M phosphate buffer solution (pH 7.2) are depicted in **Fig. 5.7.**

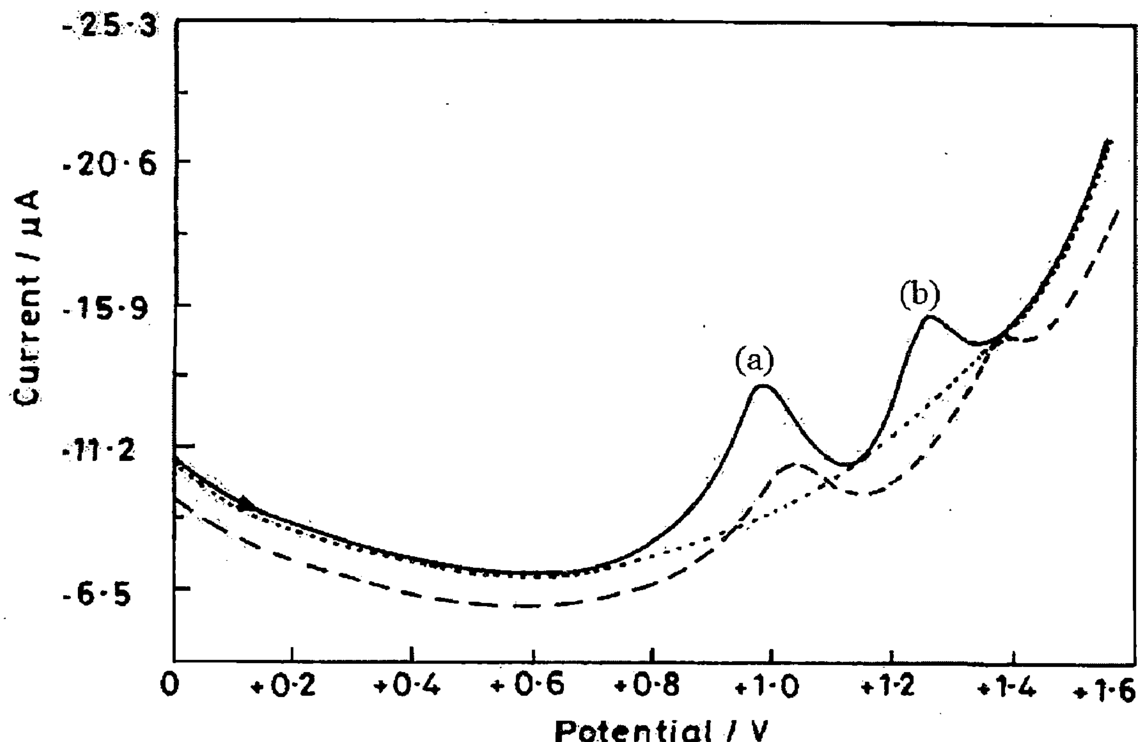


Fig. 5.7 Square wave voltammograms for a mixture of 50 μM adenine (a) and 50 μM 2'- deoxyadenosine (b) solution at pH 7.2 using fullerene – C_{60} – modified glassy carbon electrode (—) and bare glassy carbon electrode (- - - -) and, background PBS at fullerene – C_{60} – modified glassy carbon electrode (....).

At the modified electrode, (curve a), adenine and 2'-dAdo produce two well-defined oxidation peaks at 994 mV and 1248 mV respectively, while at bare GCE comparatively poor response is obtained (curve b). Since fullerene – C_{60} film exhibits excellent electrocatalytic property, the modified electrode exhibits an apparent shift of the oxidation potential to less positive potentials as compared to bare GCE. For adenine and 2'-dAdo the shift in peak potentials (ΔE_p) were ~ 40 mV and ~ 150 mV, respectively. Marked

enhancement in peak current values was also observed at modified electrode. Reduction in oxidation potential and enhancement of the peak current of oxidation peaks of analytes suggest that fullerene film acts as a very efficient promoter to enhance the kinetics of the electrochemical process by proton abstraction from molecules under investigation [49, 50]. The shift in peak potential to less positive potential clearly indicates that the fullerenes exhibit electrocatalytic effect. The electrocatalytic effect of fullerenes and carbon nanotubes has been assigned to trace amount of metals entrapped in the cavity during oxidation of number of biomolecules [2, 51].

5.3.8 Individual determination of adenine and 2'-dAdo

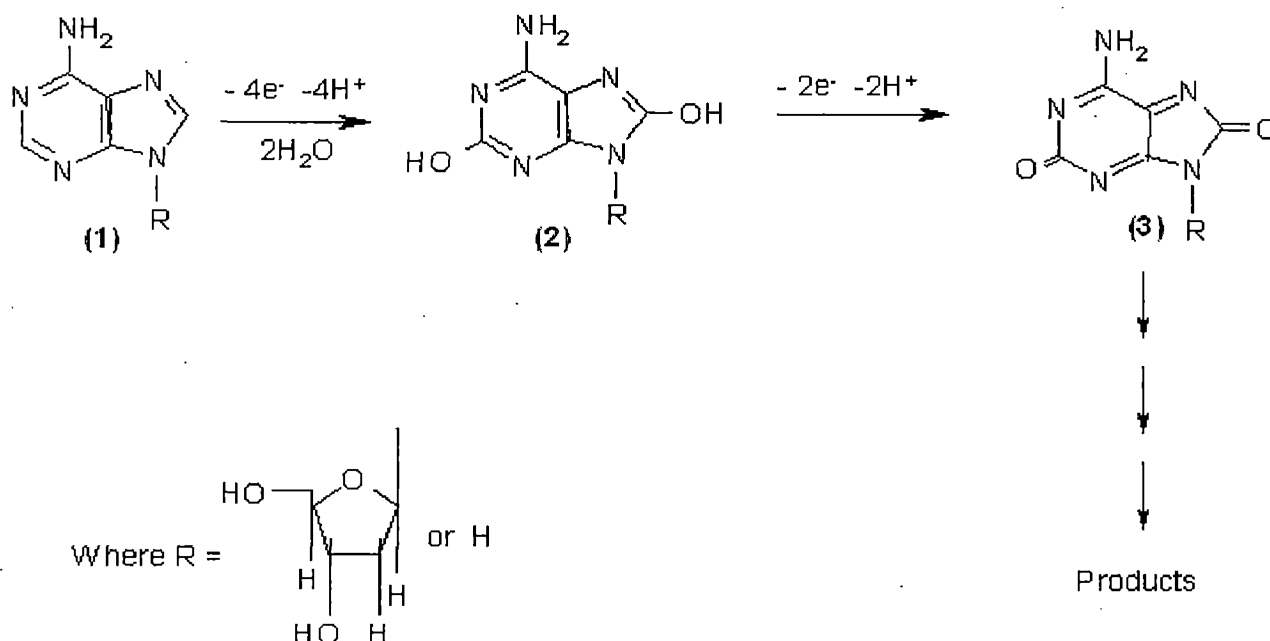
5.3.8.1 Effect of pH

In the entire pH range studied, the oxidation of adenine and 2'-dAdo occurred in a single well-defined pH dependent peak. The peak potentials of oxidation peak of both analytes shifted to less positive potential with increase in pH at modified electrode. The plots of E_p vs. pH were linear for both analytes. The values of dE_p/dpH observed for adenine (~ 64 mV/pH) and for 2'-dAdo (~ 51 mV/pH), indicate the involvement of equal number of protons and electrons in the electrode reaction for both compounds [52, 53]. The E_p vs. pH relationship can be expressed by the relations:

E_p /mV = 1461.2 – 64.55 pH versus Ag/AgCl (for adenine)

E_p /mV = 1611.7 – 51.21 pH versus Ag/AgCl (for 2'-dAdo)

with the correlation coefficient 0.9977 and 0.9847 respectively. The electrooxidation of adenine (1) and 2'-deoxyadenosine (2) has been studied earlier and it has been suggested that primary electrode reaction occurs by a loss of $6e^-$, $6H^+$ at pH 7.2 as illustrated in **Scheme 1** [54, 55].



Scheme 1. The primary electrode reaction mechanism proposed for the electro-oxidation of adenine and 2' - deoxyadenosine at pH 7.2.

5.3.8.2 Effect of square wave frequency

Square wave voltammograms (SWVs) of 50 μM adenine and 50 μM 2'-dAdo were recorded separately at modified electrode in 0.1 M phosphate buffer solution at various square wave frequencies. The peak current (i_p) of both species were directly proportional to the square wave frequency (f). The plots of i_p versus f for both analytes were linear. The linear nature of these plots suggested that adsorption plays a significant role in the electrode process [41].

The effect of square wave frequency on peak potential (E_p) of both analytes was studied in the range 5 – 200 Hz at pH 7.2. The E_p was found to shift towards more positive potentials and the plots of E_p versus $\log f$ for both analytes were linear which suggests the nature of electrode reaction as reversible in which electron transfer is coupled with a follow up chemical reaction [48, 56]. The primary electrode reaction of these compounds lead to an unstable diimine which on hydrolysis leads to number of products. The dependence of E_p on $\log f$ can be expressed by the equations:

$E_p = (68.04 \log f + 910.62)$ mV versus Ag/AgCl (for adenine)

$E_p = (50.40 \log f + 1171.2)$ mV versus Ag/AgCl (for 2'-dAdo)

with the correlation coefficient 0.9869 and 0.9932, respectively.

5.3.8.3 Calibration plots

The square wave voltammograms of adenine and 2'-dAdo in 0.1M PBS (pH 7.2) were recorded at different concentrations. The anodic peak currents increased with increase in concentration of analytes. The plots of peak current versus concentration were linear in the concentration range 10 nM to 100 μM as shown in **inset of Fig 5.8**. The linear regression equations for calibration plots of adenine and 2'dAdo can be represented as:

i_p (μA) = 0.0802 C (μM) + 0.2729 versus Ag/AgCl (for adenine)

i_p (μA) = 0.0512 C (μM) + 0.1639 versus Ag/AgCl (for 2'-dAdo)

with a correlation coefficients of 0.9947 and 0.9986, respectively. The limit of detection at pH 7.2 was calculated by the formula $3\sigma/b$, where σ is the standard deviation of blank and b is the slope of the calibration curve. The limits of detection for adenine and 2'-dAdo were found to be 0.95×10^{-8} M and 0.8×10^{-8} M, respectively.

5.3.9 Simultaneous determination of 2'dAdo and adenine

The simultaneous determination of adenine and 2'-dAdo at fullerene – C_{60} – modified GCE was carried out by keeping the concentration of one compound constant and varying the concentration of other. **Fig. 5.8 (A)** depicts the square wave voltammograms obtained at fixed concentration of adenine (50 μM) and varying the concentration of 2'-dAdo. It can be seen that i_p of adenine (fixed component) remained almost constant while the i_p of 2'-dAdo increases with an increase in its concentration. **Fig. 5.8 (B)** shows the square wave voltammograms obtained at fixed concentration of 2'-dAdo (100 μM) and varying the concentration of adenine. It was noticed that i_p of 2'-dAdo (fixed component) remained almost constant while the i_p of adenine increases with an increase in its concentration. The current observed in both the cases for varied components were essentially same as observed during the individual compound study, and obeyed the calibration plot. Therefore, using the proposed method, 2'dAdo & adenine can be easily estimated in a mixture within the concentration range of 10 nM to 100 μM with good sensitivity.

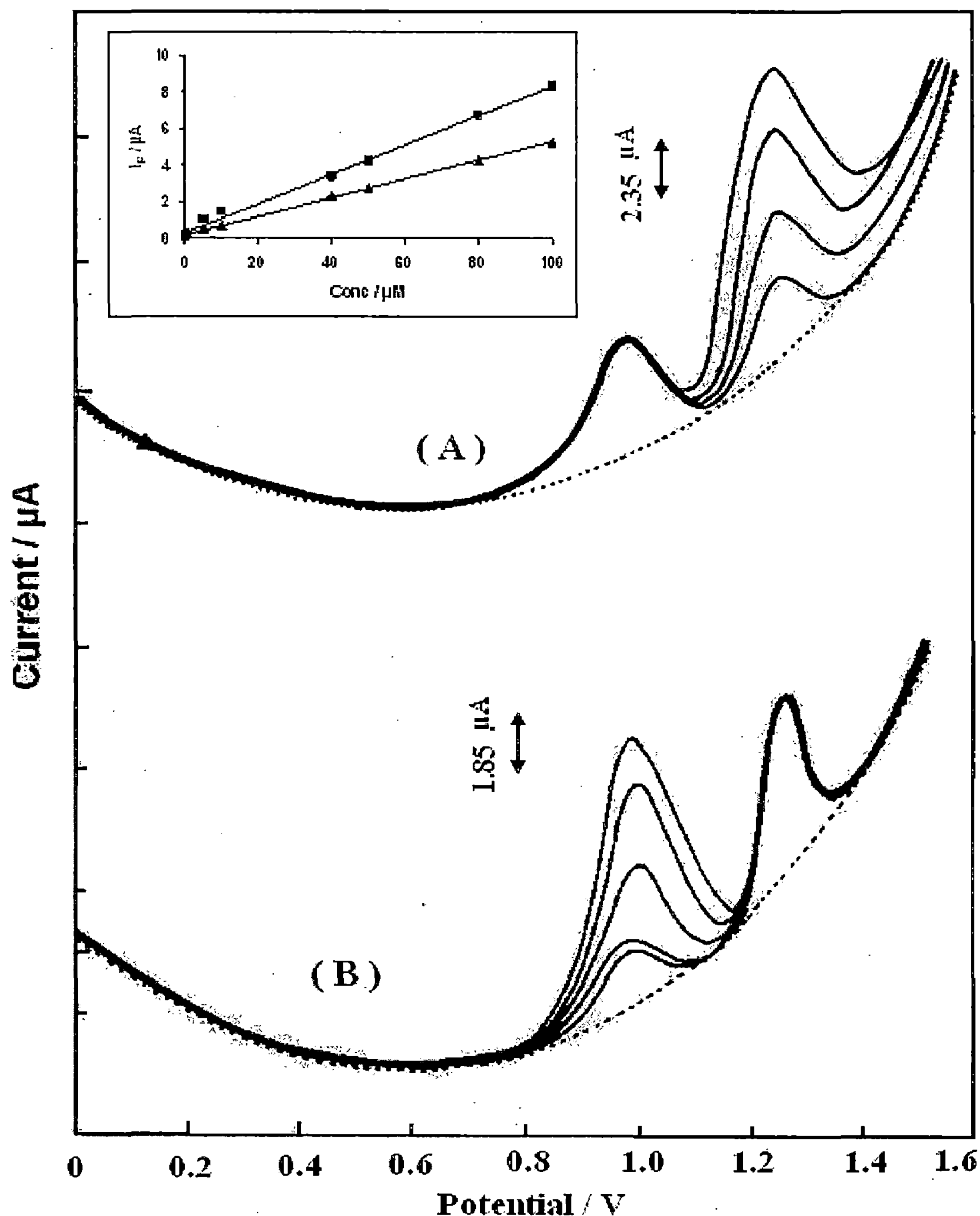


Fig. 5.8 Square wave voltammograms of a binary mixture of adenine and 2'-dAdo using C_{60}/GCE at pH 7.2. (A) Concentration of 2'-dAdo was changed keeping the concentration of adenine constant [adenine] = 0.05 mM; [2'-dAdo]: 0.05, 0.10, 0.15, 0.20 mM and (B) Concentration of adenine was changed keeping the concentration of 2'dAdo constant [2'-dAdo] = 0.1 mM; [adenine]: 0.01, 0.02, 0.05, 0.08, 0.1 mM. Calibration curves of adenine (■) and 2'-dAdo (▲) at pH 7.2 are shown in inset.

5.3.10 Effect of interferents

It was considered worthwhile to evaluate the interference of uric acid, ascorbic acid, xanthine and hypoxanthine because of coexistence of these common biomolecules with analytes (adenine and 2'-dAdo) in living systems. These species can interfere in the detection of analytes because of their close oxidation potentials. The influence of these four compounds on oxidation peak response of adenine and 2'-dAdo was observed by recording SWV for binary mixtures having constant amount (50 μM) of both the analytes (adenine and 2'dAdo) and varying the concentrations of each interferent in the range 0.05 – 0.60 mM. There was no considerable effect on peak current, when hypoxanthine was present with adenine in equimolar amount (each 50 μM). However, adenine peak broadened at the hypoxanthine concentration ≥100 μM and followed the same trend till twelve fold excess, when hypoxanthine peak started overlapping with adenine. In the case of xanthine, uric acid and ascorbic acid no substantial change in peak current response of adenine was observed for entire concentration range of interferents. In the presence of hypoxanthine, the oxidation peak of 2'-dAdo remained practically unaffected till four fold- excess (0.20 mM). However, the oxidation peak of 2'-dAdo was overlapped by hypoxanthine oxidation peak when hypoxanthine concentration was at eight fold excess and higher. The oxidation peak current of 2'-dAdo practically remains unaffected till the xanthine concentration at eight fold excess. At higher concentrations of xanthine the oxidation peak current of 2'-dAdo decreased significantly. Both uric acid and ascorbic acid did not effect oxidation peak current of 2'-dAdo in the entire concentration range studied (**Table 5.3**).

Table 5.3 Influence of interferents on the voltammetric response of mixture of 0.05 mM adenine and 0.05 mM 2'-dAdo at fullerene modified glassy carbon electrode

Interferent	Conc. (mM)	Change in peak current (μA)			
		Adenine		2'-dAdo	
		i_p	%	i_p	%
Ascorbic acid					
	0.05	+ 0.04	0.95	+ 0.028	1.00
	0.10	+ 0.04	0.95	+ 0.037	1.33
	0.20	+ 0.09	2.14	+ 0.037	1.33
	0.40	+ 0.12	2.85	- 0.036	1.29
	0.60	+ 0.12	2.85	- 0.042	1.51
Hypoxanthine					
	0.05	+0.07	1.67	+0.037	1.33
	0.10	Peak disappeared		+ 0.046	1.65
	0.20	_		+ 0.046	1.65
	0.40	_		Peak disappeared	
Uric acid					
	0.05	+ 0.00	0.00	+ 0.00	0.00
	0.10	0.06	1.43	+ 0.050	1.79
	0.20	+ 0.07	1.67	+ 0.046	1.65
	0.40	+ 0.07	1.67	+ 0.055	1.97
	0.60	+ 0.09	2.14	+ 0.065	2.33
Xanthine					
	0.05	- 0.09	2.14	+ 0.065	2.33
	0.10	- 0.08	1.90	+ 0.037	1.33
	0.20	+ 0.10	2.38	- 0.065	2.33
	0.40	+ 0.12	2.85	- 0.074	2.66
	0.60	+ 0.14	3.33	- 0.158	5.66

The influence of the interferents was also studied on a fixed concentration of adenine (0.05 mM). It was observed that except hypoxanthine no other compound showed interference. The interference of EDTA was also checked as the blood samples were collected in EDTA. It was found that addition of EDTA does not cause significant change in peak current of adenine as shown in **Table 5.4**. The effect of albumin was also studied on the voltammetric response of a mixture of 50 μM adenine and 2'-dAdo. There was no remarkable change in the peak current response of both the analytes as the concentration of albumin was varied in the range 0.1 – 5 μM.

Table 5.4 Influence of interferents on the voltammetric response of 0.05 mM adenine at fullerene modified glassy carbon electrode

Interferent	Conc. (mM)	Change in peak current (μA) Adenine	
		i_p	%
Ascorbic acid	0.05	+ 0.06	1.43
	0.10	- 0.03	0.71
	0.30	+ 0.15	3.57
	0.50	+ 0.08	1.90
Hypoxanthine	0.05	+ 0.13	3.09
	0.10	Peaks overlapped	
	0.30	–	
	0.50	–	
Uric acid	0.05	+ 0.04	0.95
	0.10	+ 0.07	1.67
	0.30	+ 0.05	1.19
	0.50	- 0.12	2.86
Xanthine	0.05	+ 0.08	1.90
	0.10	- 0.06	1.43
	0.30	- 0.09	2.14
	0.50	+ 0.13	3.09
EDTA	0.05	+ 0.04	0.95
	0.10	+ 0.12	2.86
	0.30	- 0.06	1.43
	0.50	+ 0.05	1.19

5.3.11 Analytical applications

The main aim of present study is simultaneous determination of adenine and 2'-dAdo in biological samples. The determination of 2'dAdo and adenine was performed in blood plasma and urine samples by standard addition method. Two human urine samples were collected from laboratory personnel and used as control. To minimize matrix complexity, the samples were diluted 10 times with phosphate buffer solution (pH 7.2) and then square wave voltammograms were recorded. Curve a in **Fig 5.9** presents voltammogram of background, whereas curve b presents voltammogram of control urine. Curve c presents voltammogram of urine sample of leukemic patient. Three well-defined peaks were observed in control urine at 700, 994 and 1248 mV. These peaks were assigned to xanthine, adenine and 2'-dAdo, indicating thereby that these compounds are excreted in human urine under ordinary conditions. Both control samples were then spiked with known amount of adenine and 2'-dAdo solutions and the peak current of both analytes was measured again. Curve d in Fig 5.9 presents voltammogram of control urine spiked with adenine. The concentrations of adenine and 2'-deoxyadenosine were determined by using regression equation and found as 6.5 μM and 0.20 μM, respectively in the urine samples of control.

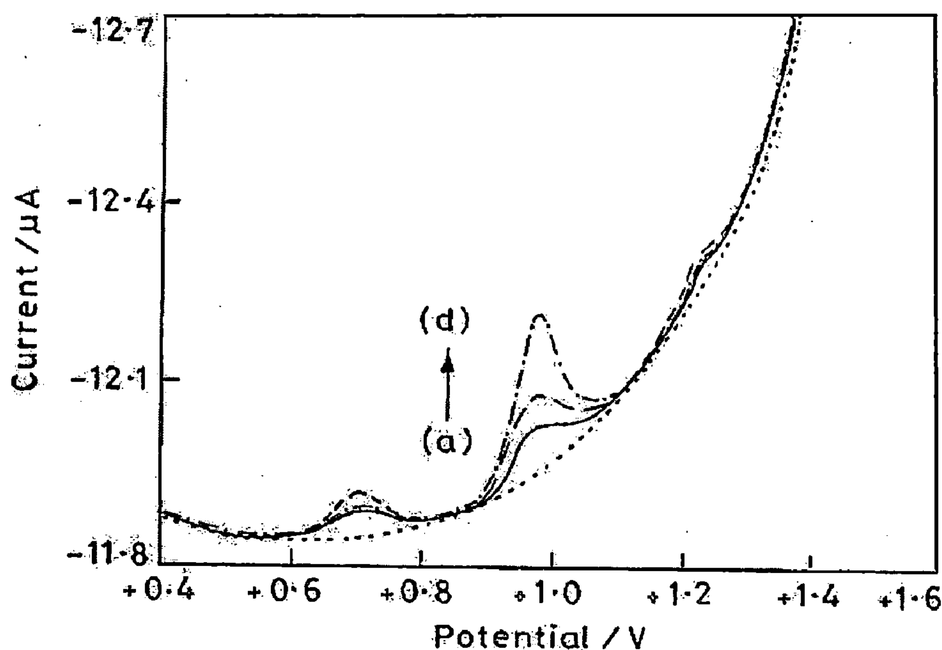


Fig. 5.9 Typical square wave voltammograms of blank PBS as background (a), control urine sample (b), urine sample of leukemic patient (c) and control urine sample spiked with adenine (d) at pH 7.2 using C_{60}/GCE.

The results of standard addition method obtained by voltammetry and HPLC clearly indicate that the amounts of 2'-dAdo and adenine present in normal human urine (control) are 0.20 μM and 6.5 μM, respectively as presented in **Table 5.5**.

Table 5.5 A comparison of recovery of adenine and 2'-deoxyadenosine added to human urine by voltammetric and HPLC methods

Added (mM)	Adenine observed (mM) by*		2'-deoxyadenosine observed (mM) by*	
	Voltammetry	HPLC	Voltammetry	HPLC
Sample 1				
0.000	0.0065 (—)	0.0064 (—)	0.0002 (—)	0.0002 (—)
0.030	0.0375 (102.74)	0.0368 (101.10)	0.0307 (101.65)	0.0309 (102.32)
0.050	0.0562 (99.47)	0.0567 (100.53)	0.0506 (100.80)	0.0504 (100.40)
0.070	0.0782 (102.22)	0.0792 (103.66)	0.0698 (99.43)	0.0722 (102.85)
Sample 2				
0.000	0.0064 (—)	0.0066 (—)	0.0002 (—)	0.0002 (—)
0.030	0.0372 (102.20)	0.0378 (103.30)	0.0309 (102.32)	0.0312 (103.31)
0.050	0.0582 (103.19)	0.0569 (100.53)	0.0506 (100.80)	0.0509 (101.39)
0.070	0.0786 (102.90)	0.0775 (101.17)	0.0708 (100.85)	0.0731 (104.13)

* Recovery % has been mentioned in parentheses. The RSD was < 2.5% for n = 3.

Three human blood plasma samples were obtained from the Hospital of IIT Roorkee and each sample was diluted 10 times with 0.1 M phosphate buffer solution of pH 7.20. The voltammograms of plasma samples did not exhibit peaks corresponding to adenine or 2'dAdo as their concentration level is very low. The analysis was then carried out using standard addition method and recovery tests of both analytes in blood plasma samples were performed in the concentration range 0.05 mM to 0.10 mM by voltammetry and HPLC methods. The observed recoveries were 98.00 % to 104.00 % for adenine and 96.00 % to 104 % for 2'-dAdo that is comparable to HPLC results as shown in **Table 5.6.**

Table 5.6 A comparison of analytical recovery of adenine and 2'-deoxyadenosine added to human blood plasma by voltammetric and HPLC methods

Added (mM)	Adenine observed (mM) by *		2'-deoxyadenosine observed (mM) by *	
	Voltammetry	HPLC	Voltammetry	HPLC
Sample 1				
0.050	0.051 (102.00)	0.052 (104.00)	0.051 (102.00)	0.051 (102.00)
0.080	0.082 (102.50)	0.081 (101.25)	0.079 (98.75)	0.078 (97.5)
0.100	0.102 (102.00)	0.101 (101.00)	0.102 (102.00)	0.101 (101.00)
Sample 2				
0.050	0.049 (98.00)	0.051 (102.00)	0.048 (96.00)	0.049 (98.00)
0.080	0.083 (103.75)	0.082 (102.50)	0.081 (101.25)	0.082 (102.50)
0.100	0.101 (101.00)	0.102 (102.00)	0.097 (97.00)	0.098 (98.00)
Sample 3				
0.050	0.051 (102.00)	0.052 (104.00)	0.052 (104.00)	0.051 (102.00)
0.080	0.083 (103.75)	0.083 (103.75)	0.082 (102.50)	0.081 (101.25)
0.100	0.099 (99.00)	0.098 (98.00)	0.100 (100.00)	0.101 (101.00)

* Recovery % has been mentioned in parentheses. The RSD was < 2.2 % for n =3.

The practical utility of the present method was over again examined by analyzing urine sample from patients suffering from hepatocellular carcinoma. For this purpose, voltammograms of urine samples of patients were recorded using modified electrode. In order to reduce matrix complexity, urine samples were diluted 10 times with addition of 0.1 M phosphate buffer solution (pH 7.2). The voltammogram recorded for urine sample of carcinoma patient shows a large peak at the same potential as for the adenine and a small peak at potential corresponding to 2'-dAdo as shown in curve c of Fig.5.9. The peak current indicated adenine concentration as 1.25 μM and 2'-dAdo concentration as 0.035 μM. As the urine sample was used after 10 times dilution, hence, the concentration of adenine and 2'-dAdo in urine sample of carcinoma patient was calculated as 12.50 μM and 0.35 μM, respectively. The comparison of concentration of adenine and 2'-dAdo in normal person

and patient urine samples determined by voltammetric method has been presented as column graph in **Fig. 5.10**. The higher concentration of adenine in urine sample of leukemic patients is well reported in literature [38].

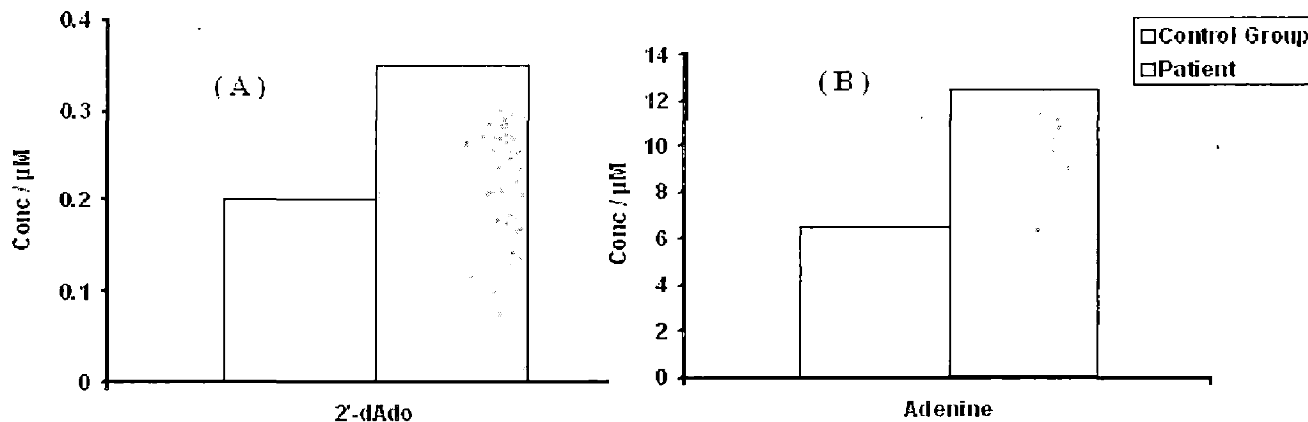


Fig. 5.10 A comparison of observed concentration of 2'-dAdo (A) and adenine (B) in the human urine of control group and patients determined by voltammetry.

5.3.12 Comparison of results by HPLC

The proposed method has also been validated by using high performance liquid chromatography. For cross validation different concentrations of adenine and 2' -dAdo were analyzed using HPLC and well defined peaks were obtained at retention time, R_t ~ 4.120 min and R_t ~ 5.063 min. Calibration plots were obtained by plotting the peak area versus analyte concentration. The resulting calibration plots were linear. Curve A in **Fig 5.11** presents chromatogram of adenine sample and curve B presents control urine sample. The peak at R_t ~ 4.120 min is of adenine and the peak at R_t ~ 5.063 is found to be due to 2'-dAdo. However, no attempt was made to identify rest of the peaks which may be likely due to uric acid and xanthine. To confirm that peak at R_t 4.120 min is due to adenine, a chromatogram was also recorded after spiking control urine with adenine as shown in curve C. The HPLC chromatograms of diluted urine samples from carcinoma patients were then recorded. Curve D in Fig. 5.11 depicts HPLC chromatogram observed for urine sample of a patient suffering with hepatocellular carcinoma. The adenine and 2'-dAdo concentrations of undiluted urine sample from HPLC were found as 12.4 µM and 0.36 µM, respectively which are in good agreement with that obtained using proposed method. The correlation between electrochemical and chromatographic detection has been shown in **Table 5.7**.

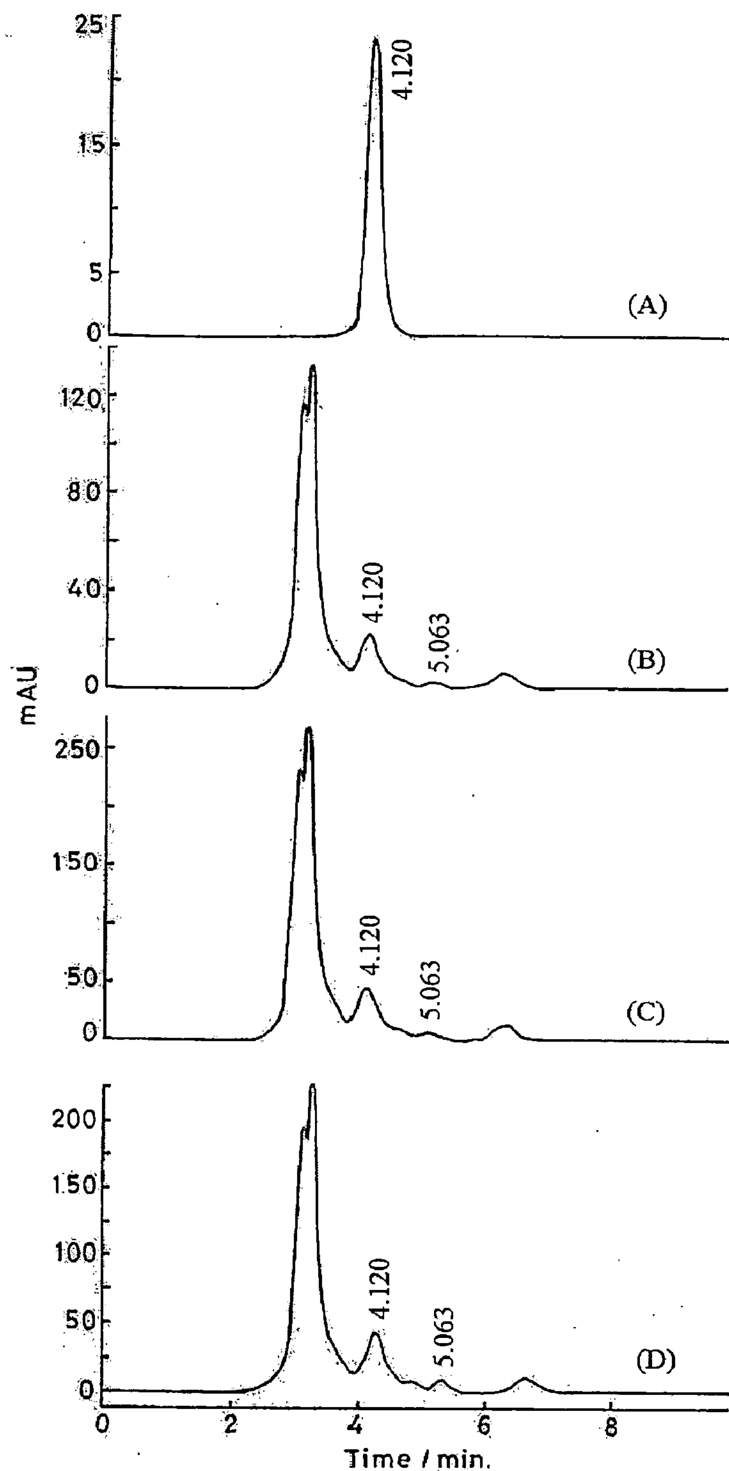


Fig. 5.11 Observed HPLC chromatograms of (A) standard adenine, (B) normal human urine used as control, (C) adenine spiked in normal human urine and (D) urine sample of a leukemic patient.

Table 5.7 Correlation between electrochemical and chromatographic detection

Sample	Adenine observed (μM)*		2'-deoxyadenosine observed (μM)*	
	Voltammetry	HPLC	Voltammetry	HPLC
Control Urine	6.50	6.40	0.20	0.20
Patient Urine	12.50	12.40	0.35	0.36

* The RSD was < 3.1 % for n =3.

5.3.13 Reproducibility and stability of modified electrode

Six consecutive square wave voltammograms were recorded separately for adenine (50 μM) and 2'-dAdo (50 μM) at same fullerene – C_{60} – modified GCE. It was found that modified electrode shows good reproducibility with relative standard deviation (R.S.D) of 3.2 % for adenine and 2.5 % for 2'-dAdo. To examine electrode to electrode variation response, six glassy carbon electrodes (dia ~3 mm) were casted with 40 μL of fullerene solution. It was found that these electrodes exhibit a variation of ± 3.5 % in peak current of 50 μM adenine and 2'-dAdo. Thus, it is concluded that the electrode to electrode variation is non-significant.

The stability of modified electrode is not so important because modification method and cleaning process are easy and also less time consuming. It was stored in air at room temperature. Up to four days of its preparation, it shows negligible changes in response in spite of continuous use. After five days oxidation peak potentials shifted towards positive potentials and current values were also decreased probably because of disintegration of fullerene film. Therefore, the prepared electrode can be easily used up to five days of its preparation.

5.4 CONCLUSIONS

The present study described the use of fullerene – C_{60} – modified electrodes for the determination of biomolecules and drugs of biological relevance. It has also been clearly demonstrated that with the successive removal of metallic impurities from fullerene peak potential of nandrolone shifts to more positive values and there is a marked decrease in peak current. Thus, the catalytic activity of fullerene towards the oxidation of nandrolone appears to be due to the embedded metallic impurities that are accessible to fluids, present in it and further purification of fullerene simply decreases the peak current and leads to decrease in sensitivity which is not desirable for analytical purposes. Hence, it is advocated that fullerene of purity more than 98 % can be used directly for electroanalytical applications avoiding any complex and time consuming purification procedure. It is also concluded that edge plane pyrolytic graphite electrode serves as the best substrate for fullerene modification in comparison to other conventional electrodes like ITO, gold, GCE and BPPGE. The use of EPPGE is thus advantageous for electrochemical determinations because of its highly reactive edge plane sites which allow low overpotentials, high sensitivity, low detection limit and improved signal to noise characteristics [57].

Another vital aspect of present studies is the use of fullerene – C_{60} – modified glassy carbon electrode to determine adenine and 2'-deoxyadenosine in human blood plasma and urine samples. In contrast to other reported approaches present method requires less sample amount, financial input and permits combined analysis of nucleosides and nucleobases. Due to metabolic disorders like severe combined immunodeficiency and cancer the level of these biomolecules in blood plasma and urine is elevated to some extent. Hence, C_{60} modified GCE can be utilized satisfactorily to detect such diseases.

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