DEVELOPMENT OF VOLTAMMETRIC SENSORS FOR THE DETERMINATION OF BIOMOLECULES AND DOPING AGENTS

A THESIS

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CANDIADATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled, **"DEVELOPMENT OF VOLTAMMETRIC SENSORS FOR THE DETERMINATION OF BIOMOLECULES AND 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, 2009 to October, 2013 under the supervision of Dr. R.N. Goyal, Professor, Department of Chemistry, Indian Institute of Technology Roorkee, Roorkee.

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

(BHARATI AGRAWAL)

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

(R.N. Goyal)
Date: Supervisor

The Ph.D. Viva-Voce Examination of **Ms. Bharati Agrawal**, Research Scholar, has been held on ……..

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ABSTRACT

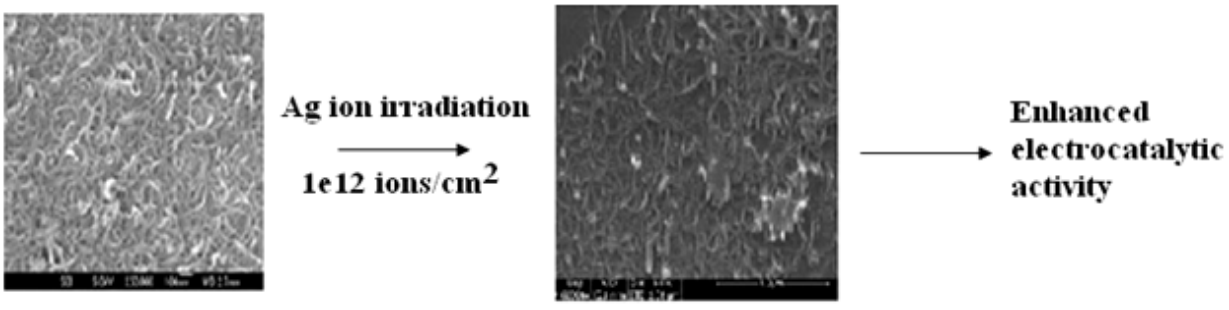
Over the last decade numerous developments in nanoscience and nanotechnology have contributed significantly to the electrochemistry. Nanotechnology based electrochemical platform offers a promising tool for attainment of multiple aims in biomolecular analysis. Nanomaterials prepared from metal, semiconductors and carbon or polymeric species have allured great attention due to their widespread applications in different areas of science. There has been a substantial progress in construction of highly efficient nanomaterials based electrochemical sensors for monitoring of biologically important molecules and pharmaceutical drugs. It is observed that the sensitive and selective detection of specific biomolecules and drugs is mandatory for elucidating the physiological processes as well as for early diagnosis and therapy of diseases. The recent upcoming of new forms of carbon based nanomaterials such as fullerene, graphene and carbon nanotubes (CNT) have revolutionalized the electrochemical research and brought many potential applications in nanoscience. These carbon based nanomaterials have enticed many researchers due to their attractive electronic, optical, thermal and electro-catalytic properties over other conductive materials. These properties together with their nanometric size and high aspect ratio make them suitable for electrochemical sensing of verities of organic compounds. Considering the significance of nanomaterials in the area of electrochemistry, in this thesis, an attempt has been made to systematically utilize the different modification approaches employing carbon based nanomaterials with a focus on the development of highly sensitive electrochemical sensors for the investigations of biomolecules and doping agents. The thesis is divided in six chapters.

In **chapter 1** an overview on conventional electrodes, types of nanomaterials and modification of electrodes using nanomaterials with particular emphasis on carbon nanotubes modified electrode is presented. This chapter also deals with the illustration of methodology employed in the present investigation comprising some theoretical aspects of voltammetric techniques.

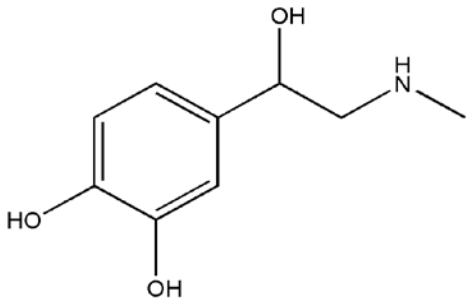
Chapter 2 describes the application of multi-walled carbon nanotubes (MWCNT) modified edge plane pyrolytic graphite electrode (EPPGE) for studying the electrochemical reduction of Norfloxacin (NFX). The modified electrode was subjected to the determination of NFX in biological fluids and pharmaceutical tablets. The modified electrode exhibited excellent electro-catalytic properties towards the reduction of NFX by decreasing the reduction peak potential and

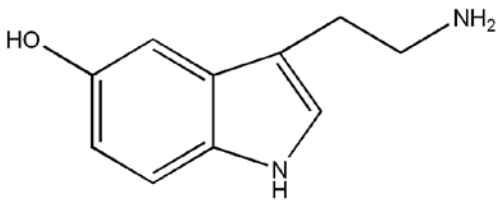
increasing the cathodic peak current. Moreover, nanotubes modified electrode was also used to investigate the effect of NFX on catabolism of caffeine through its determination in urine sample. It is found that the long term administration of NFX reduces the catabolism of caffeine by preventing its demethylation, which may lead to accumulation of caffeine in human system. It is suggested that patient on medication with NFX should avoid the excess consumption of caffeine. This is the first time detection of NFX using nanotubes modified electrode based on its electrochemical reduction. The advantage of present method for detection of NFX is the large negative potential window at which the common metabolites of urine do not interfere with the analyte due to their non-reducible nature. The modified electrode determined the NFX concentration ranged between 1.2 and 1000 µM with detection limit (3σ/slope) and sensitivity of 40 ± 3.3 nM and 0.072 µA μM^{-1}, respectively. The method is simple, sensitive and can be readily applied to monitor the NFX in urine samples and also its effect on catabolism of caffeine.

Chapter 3 deals with the use of concept of swift heavy ion irradiation of carbon nanotubes and its effect on structural properties of nanotubes. MWCNT was irradiated by Ag ions of high energy (~ 120 MeV) at different fluences of 1e12, 3e12 and 1e13 ions cm^{-2} with 15 UD Pelletron Accelerator. After optimizing the experimental parameters, the irradiated sensor was employed for the simultaneous determination of two important neurotransmitters; Epinephrine and 5-Hydroxytryptamine (serotonin). Simultaneous determination of both neurotransmitters was carried out in phosphate buffer of pH 7.2 (as supporting electrolyte) using square wave voltammtery (SWV) and cyclic voltammetry (CV).



[Nanotubes before and after heavy ion irradiation]



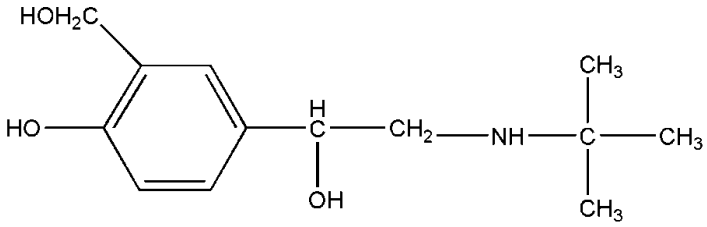


[Epinephrine] **[Serotonin]**

Irradiation by Ag ions caused an enhancement in electrocatalytic activities of nanotubes due to the increased conductivity with insertion of Ag ions and an increment in effective surface area after irradiation. The irradiated sensor was applied for the determination of epinephrine and serotonin in real samples such as blood and urine and exhibited good accuracy and precision of the method. The coexisting metabolites present in urine such as uric acid, ascorbic acid and dopamine did not show any interference during the simultaneous determination of epinephrine and serotonin.

In **chapter 4**, the application of single-walled carbon nanotubes (SWCNT) for the modification of EPPGE is documented. The modified sensor was used for electrochemical reduction of a topical corticosteroid; Halobetasol propionate (HBP). At SWCNT-coated EPPGE, the electrochemical response of HBP increases significantly as reduction peak current increases and peak potential shifts to less negative direction as compared to bare EPPGE, showing the electrocatalytic ability of SWCNT towards the reduction of HBP. The controlled potential electrolysis was performed to obtain the product of HBP formed during its electrochemical reduction. For this purpose, the solution of HBP was electrolyzed by applying constant potential ~ 70 mV more negative than reduction peak potential of HBP using potentiostat. The product of reduction was characterized by FT-IR and ^{1}H-NMR spectroscopic measurements and the possible site of reduction was deduced as >C=O. The proposed methodology was also used for the determination of HBP in various pharmaceutical preparations. The results obtained from the determination of HBP in tablet samples did not show any interference from the excipients, viz KCl, NaCl and petroleum jelly because all these compounds are not reducible. The modified electrode showed good stability and reproducibility with relative standard deviation of 2.21 % and 3.32 %, respectively, confirming that this approach can be successfully used for the determination of HBP in various pharmacological samples.

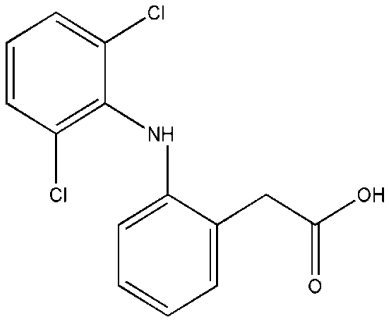
Chapter 5 illustrates the utilization of SWCNT modified EPPGE for the investigation of an important β_2-agonist; Salbutamol, which is used as bronchodilator in the treatment of asthmatic disorders and chronic obstructive pulmonary diseases. Salbutamol is found to be able to increase muscle protein, reduce total body fat and promote muscle growth, hence, it is abused by athletes in competitive games. Its use in sports by athletes has been banned by World Anti Doping Agency (WADA) and it has been listed as a doping agent. The threshold concentration of this drug is 1000 ng mL^{-1}, and gives an indication of oral administration according to WADA rules. In the proposed work, modified electrode showed improved voltammetric response towards the oxidation of salbutamol with well-defined peak at ~ 600 mV with enhanced peak current in comparison to bare electrode. The CNT increased the electrochemical performance of electrode due to their excellent conductivity and high surface area. The proposed sensor showed a good linear range, low detection limit, high sensitivity with good stability and reproducibility. The sensing of salbutamol was carried out in pharmaceutical tablets and human body fluids which make the proposed method of significant interest for doping control purposes at the site of competitive games.

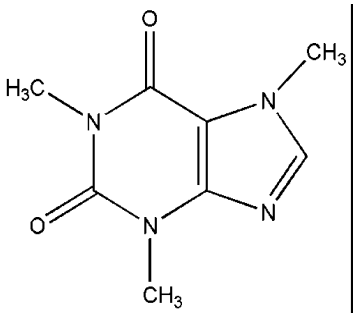


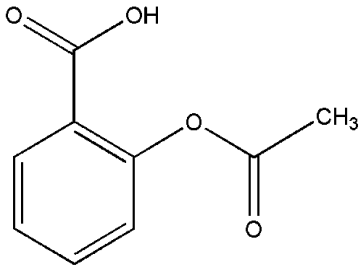
[Salbutamol]

The last chapter of thesis **chapter 6** describes the application of EPPGE for the determination of two analgesic drugs. In the first section of this chapter, the electrochemical study of non-steroidal anti-inflammatory drug; Diclofenac has been presented. Diclofenac has analgesic, antipyretic and anti-inflammatory properties and is widely prescribed in clinical medicine for the treatment of several diseases. The investigation of this drug was carried out at EPPGE using SWV and CV in phosphate buffer of pH 7.2. The oxidation peak current increased linearly with the concentration of diclofenac in the range 10 – 1000 nM and detection limit and sensitivity of proposed method were 6.2 nM and 69 nA nM^{-1}, respectively. The controlled potential electrolysis was performed and the product formed from the oxidation of diclofenac was characterized using

^{1}H-NMR. The developed method was applied for the determination of diclofenac in pharmaceutical formulation and urine samples obtained from the patients undergoing treatment with diclofenac.







[Diclofenac] **[Caffeine]** **[Aspirin]**

The second section of this chapter presents the results on the determination of two analgesic drugs; Caffeine and Aspirin. Caffeine is a stimulant drug which arouses the central nervous system and cardiovascular system. It is prescribed as an analgesic adjuvant in pharmaceutical preparations for the treatment of headache and pain. Caffeine is used by professional athletes to give them alertness and extra energy for their work so that they could improve their physical performance. Therefore, caffeine has been reported as a doping agent at a level of 12 µg mL^{-1} by WADA. Aspirin is an important analgesic that reduces the pain without interfering the functions of other sense organs. Bare EPPGE was used for the determination of caffeine and aspirin at pH 7.2. The electrode showed two well-defined peaks having peak potential of ~ 1225 mV and ~ 1335 mV for aspirin and caffeine, respectively. The peak current of oxidation peaks was found to increase with increase in the concentration of caffeine and aspirin in the range 0.02 – 100 µM and detection limits of 0.01 µM and 0.08 µM, respectively are observed. The proposed sensor was successfully applied for the determination of caffeine and aspirin in urine samples, pharmaceutical preparations and coffee beverages.

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Dated: **BHARATI AGRAWAL**

LIST OF PUBLICATIONS

1. Rajendra N. Goyal, **Bharati Agrawal**, "Ag ion irradiated based sensor for the electrochemical determination of epinephrine and 5-hydroxytryptamine in human biological fluids", **Analytica Chimica Acta** 743 (2012) 33-40.

2. Rajendra N. Goyal, **Bharati Agrawal**, "Carbon nanotubes-based electrochemical sensor for the determination of halobetasol propionate, a topical corticosteroid", **Journal of Applied Electrochemistry** 42 (2012) 31-39.

3. Rajendra N. Goyal, Sunita Bishnoi, **Bharati Agrawal**, "Single-Walled-Carbon-Nanotubes-Modified Pyrolytic Graphite Electrode Used as a Simple Sensor for the Determination of Salbutamol in Urine", **International Journal of Electrochemistry** doi:10.4061/2011/373498.

4. **Bharati Agrawal**, Pranjal Chandra, Rajendra N. Goyal, Yoon-Bo Shim, "Detection of norfloxacin and monitoring its effect on caffeine catabolism in urine samples", **Biosensors and Bioelectronics** 47 (2013) 307–312.

5. Rajendra N Goyal, Sunita Bishnoi, **Bharati Agrawal**, "Electrochemical sensor for the simultaneous determination of caffeine and aspirin in human urine samples", **Journal of Electoanalytical Chemistry** 655 (2011) 97-102.

6. Rajendra N. Goyal, Sanghamitra Chatterjee, **Bharati Agrawal**, "Electrochemical investigations of diclofenac at edge plane pyrolytic graphite electrode and its determination in human urine", **Sensors and Actuators B** 145 (2010) 743–748.

LIST OF CONFERENCES ATTENDED

1. A paper entitled "Determination of Salbutamol in human body fluids and pharmaceuticals using nano-tubes modified sensor" was presented in an International conference (theme: Analytical Science for Advanced Materials Processing and Environmental Impact Assessment) organized by **International Congress on Analytical Science**, being held on 24th to 27th November, 2010 at Cochin (Kerala, India).

2. A paper entitled "Voltammetric determination of Salbutamol in human urine and pharmaceutical preparations using SWCNT/EPPGE" was presented in 4th International conference on "Recent Trends in Instrumental Methods of Analysis" being held on February 18th to 20th, 2011 in **Department of Chemistry, IIT Roorkee** (Uttarakhand, India).

3. A paper entitled "Carbon nano-tube modified sensor for the sensitive and selective detection of Norfloxacin in urine samples" was presented in fifth triennial International conference on "Advances and Recent Trends in Electrochemistry" organized by **Indian Society of Electro-analytical Chemistry (BARC, Mumbai)** from January 16th to 20th, 2013, at Ramoji Film City (Hyderabad, India).

LIST OF ABBREVIATIONS

BAS	Bio Analytical System
PGE	Pyrolytic Graphite Electrode
EPPGE	Edge Plane Pyrolytic Graphite Electrode
BPPGE	Basal Plane Pyrolytic Graphite Electrode
GCE	Glassy Carbon Electrode
ITO	Indium Tin Oxide
CNT	Carbon Nanotubes
SWCNT	Single Walled Carbon Nanotubes
MWCNT	Multi Walled Carbon Nanotubes
SHI	Swift Heavy Ion Irradiation
Ag/AgCl	Silver-Silver Chloride
CV	Cyclic Voltammetry
SWV	Square Wave Voltammetry
CPE	Controlled Potential Electrolysis
HPLC	High Performance Liquid Chromatography
FE-SEM	Field Emission Scanning Electron Microscopy
EDAX	Energy dispersive X-ray analysis
NMR	Nuclear Magnetic Resonance
FT-IR	Fourier Transform Infra Red
E_p	Peak Potential
i_p	Peak Current

f	Square Wave Frequency
v	Scan Rate
δ	Chemical Shift
LOD	Limit of Detection
LOQ	Limit of Quantification
RSD	Relative Standard Deviation
NSAIDs	Non-Steroidal Anti Inflammatory Drugs
WADA	World Anti Doping Agency
DMF	Dimethyl Formamide
NFX	Norfloxacin
EP	Epinephrine
5-HT	5-Hydroxy Tryptamine
HBP	Halobetasol Propionate
ASA	Acetyl Salicylic Acid
CAF	Caffeine

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Chapter 1

General Introduction

1.1 BACKGROUND

Electrochemistry is a field of chemistry that encompasses the inter-relations of electrical and chemical phenomena. Electrochemistry deals with the chemical reactions involving the transfer of electric charge across the electrified interface between electronic conductor (the electrode: a metal or a semiconductor) and an ionic conductors (solutions, melts and solid electrolyte). Electrochemical phenomena constitute the basis of innumerable processes in physical world in many modern technologies and they are absolutely mandatory for all living beings.

History

The foundation of electrochemistry was laid in 1791, when Luigi Galvani was examining the impacts of atmospheric electric discharge on a frog. The pioneering research in this field was begun by Michael Faraday in 1832, after introducing a new electrochemical terminology (electrode, cathode, anode, etc.) and two laws of electrolysis. In 1836, John Daniell's research for the development of primary battery took place which was the first battery to provide constant and reliable current over a long period of time. Battery research today has acquired a significant renaissance as various products such as cellular phones, laptops, computers and medical devices rely upon the light weight and long lasting batteries. In 1888, Walther Hermann Nernst, the architect of modern electrochemistry, elicited a theory connecting the electromotive force to the energy of a chemical reaction in an electrochemical cell and derived his famous equation known as "Nernst equation".

By the early twentieth century, around 1923, Prague emerged into the "Mecca of electrochemistry" when Jaroslav Heyrovsaky, called the "father of electro-analytical chemistry" recorded electro-capillary measurements with dropping mercury electrode. This manifested the advent of polarography with the swift augmentation of electroanalysis and culminated the fundamentals of all voltammetric methods [1-6].

Electro-analytical chemistry

The utilization of electrochemistry to analytical chemistry commonly includes the assessment of some electrical properties under the conditions, which directly or indirectly let the association between the property measured and the concentration of some distinct chemical species. Electrochemical techniques are routinely employed in analytical chemistry and have undergone numerous considerable developments in recent decades. Electrochemical techniques are

widely applied in various studies, monitoring of industrial materials and biologically important molecules and are the most versatile of all trace pharmaceutical drug analysis. Electrochemical methods are facile to adopt as difficulties can be resolved associated with biomedical and pharmaceutical research with high degree of precision, accuracy, sensitivity and selectivity, often in spectacularly reproducible way. Electrochemical methods propose some distinct benefits to analytical chemists, such as (1) no requirement of sample pretreatment; (2) rapidity of analysis; (3) high sensitivity and specificity; (4) comparable or better accuracy; (5) capability to assess trace and ultra trace analyte level (6) relatively inexpensive instrumentation and low price of chemical used; and (7) confined use of environmental unfriendly solvents. Furthermore, direct electrochemical analysis without involving pre-separation steps, makes possible the determination of drug content in blood, tablets or injection liquids as well as the determination of toxic substances in environmental samples as oxygen in air or in gas mixtures [7-10].

Electrochemical sensors

In last few years an attention has been paid for developing electrochemical-sensing devices using electrochemical approaches for determination of biomolecules and doping agents in pharmaceutical formulations and in human physiological fluids. A valuable definition for electrochemical sensor is “a sensor is a device that as the results of electrochemical interaction or process between analyte mixture and sensing device, transforms electrochemical information of a quantitative or qualitative forms into an analytical beneficial signals”. The concern in developing such devices is due to their inherent specificity, rapidity of response, high sensitivity, wide potential window, low background current, high chemical and electrochemical stabilities, excellent resistance to corrosion, morphological and micro-structural stability at extreme anodic and cathodic potentials and simplicity of preparation of these devices. More recently, electrochemical methods are alluring more attention towards the technique of preparation of pharmaceutical materials introducing the basic principles of green chemistry. The growing application of pharmaceuticals is causing a severe environmental problem as both via human and animal urinary and fecal excretion and pharmaceutical manufacturing discharge. The utilization of electrochemical sensors can be an option to measure the concentration of organic compounds (e.g. pharmaceuticals) and some specific ions in water matrices using ion selective electrodes and these methods are more advantageous than conventional methods [11-14].

Biological relevance of electrochemistry

Electrochemistry imparts a convenient approach for analyzing the redox chemistry of molecules which is relevant to redox reactions occurring in biological processes and defined as bioelectrochemistry. Bioelectrochemistry is a part of science in which investigation of the process of biological relevance is carried out using principles of electrochemical techniques. Investigation of electrochemical behavior of biomolecules provides some beneficial information on the role which is played by these molecules in biological processes. There is definite set of similarities observed between electrochemical ad biological reactions, such as

1) Both reactions take place at same pH and also in the presence of similar ionic strength of electrolyte.
2) Electrochemical process occurs at electrode-solution interface, whereas biological process occurs at enzyme-solution interface.
3) Both reactions can effectively occur at physiological temperature of 37^{o}C.
4) In both reactions substrate molecule has to be oriented in a specific fashion for enabling electron transfer at electrode or at active sites of enzyme.
5) Both processes can take place effectively in non aqueous conditions.

Thus, the sufficient superficial similarities between electrochemical and biological reactions warrant extensive electrochemical study of some biologically important molecules [15-17].

Biomolecules are the organic compounds that are produced by the living organism and are of great use due to their metabolic and biological effects in human system. Determination of these molecules in the area of biomedical research has become important due to the facts that a minute change in the concentration of these analytes in body fluids can alter their activities and cause various diseases, hence their analysis is highly desirable [18].

Doping agents also termed as performance enhancing drugs are the substances, which are often used by the atheletes to enhance muscular strength, mental stamina and physical performance in competitive games. These are the prohibited substances which are banned due to their strong adrenergic stimulations. The prohibited list (206 classified doping agents) of doping agents presented by World Anti Doping Agency (WADA) covers nine pharmaceutical classes of these substances (e.g., stimulants, diuretics, anti-estrogens), three forbidden doping methods (e.g., enhancement of oxygen transfer, chemical and physical manipulation and gene doping) and two groups of analytes prohibited in specific activities (e.g., alcohol and beta-blockers). To ensure the

consistency of evaluation of these prohibited substances at various doping control laboratories, WADA establishes a minimum detection capability for testing methods called “minimum required performance limit” (MRPL). Therefore, it is desirable to develop an electro-analytical method that reduces the analysis time without sacrificing analytical information. Such a method is likely to meet an essential need of monitoring doping cases [19-21].

Voltammetry is the common name assigned to the group of electroanalytical methods in which current that flows through an electrochemical cell, is evaluated as a function of applied potential and polarization is enhanced using “microelectrodes” as working electrodes having surface area of few square micrometers to square millimeters. Voltammetry has been developed from polarography (developed by Heyrovsky) which is a particular type of voltammetry and is widely used for the qualitative and quantitative analysis of heavy metals and some organic compounds in solution. A voltammetric sensor assays the concentration effect of the analyzing material based on the current-potential behavior of the reduction or oxidation reaction involved. There are numerous compounds which are found biologically active and are involved in oxidation-reduction processes can be easily determined based on their reduction and oxidation. The analytical advantages of various voltammetric techniques include excellent sensitivity with a large concentration range for both inorganic and organic species, rapid analysis time and simultaneous determination of several analytes at the same time which prove them as an effective tool for the analysis of complex mixture. Voltammetric techniques play a crucial role in pharmaceutical and biomedical analysis with the scope of drug analysis including the investigation of biological samples containing the drug and their metabolites, providing the contribution to maximal efficacy and safety of drug therapy and maximal economy of the drugs production of pharmaceuticals [22-25]. Therefore, it is considered worthwhile to construct efficient voltammetric sensors for the determination of biomolecules and doping agents in pharmacological formulations or in biological samples such as human urine or blood plasma.

1.2. LITERATURE REVIEW

A literature review regarding the conventional electrodes, nanomaterials and modified electrodes is being presented in following sections:

1.2.1 Conventional Working Electrodes used in Voltammetry

One of the ways to improve sensitivity and selectivity in electro-analytical measurements is the development of electrochemical sensor based on the materials that enable more efficient separation and determination of selected component. The fundamental procedure in an electrochemical reaction is the exchange of electrons; the exchanged electron is transferred to electrode if the reaction occurs at electrode or more precisely in double layer environment between electrode-electrolyte interface. In case of cathodic reaction, electrons are delivered from working electrode and in anodic reaction electrons are accepted by the working electrode. The working electrode comprises the most important part of an electrochemical cell. It provides an interfacial region (the solution adjacent to electrode surface) between electrode and solution where electrochemical reaction of interest occur. Working electrode is used in conjunction with auxiliary and reference electrode.

The selection of optimal working electrode is critical issue for the success of electrochemical reaction, which depends on several factors involving the usable applied potential window, participation of electron in electrode reaction and the kinetic of electron transfer reaction. The working electrode material must be an electronic conductor and should exhibit favorable redox reaction with the analyte. It must be electrochemically inert (i.e. should not generate a current itself in response of applied potential) over a wide potential window in a given electrolyte solution, prior to allow for the greatest degree of analyte characterization. Additionally, there must be an ease of surface renewal of working electrode to prevent toxicity and to remove adsorbed materials in order to achieve best electroanalytical performance [26, 27]. A wide variety of working electrodes are now available. The choice of material depends upon the potential window required (e.g. mercury electrode works only in negative direction due to the oxidation of mercury at positive potential of + 0.4 V) and as well as the rate of electron transfer which can vary from one material to another. Commonly used working electrode materials are made of carbon such as carbon paste, carbon fiber, doped carbon, glassy carbon and pyrolytic graphite. Other metals such as mercury, platinum, silver, gold, gold amalgam and semiconductor (e.g. indium tin oxide) have also been reported as electrode materials in electrochemistry based research. The general aspects of some electrode materials used in the electrochemical studies are presented below:

Indium tin oxide electrode

One of the semiconductors, indium tin oxide (ITO) is a well known electrode material and is composed of indium oxide (In_2O_3) and tin oxide (Sn_2O_3) deposited on a solid substrate (e.g. glass). The thin films of ITO are most commonly deposited on a surface by physical vapor deposition method. Its conductivity is basically due to the high Sn content within In_2O_3 crystalline lattice. Some indium atoms present in bulk oxide structure is substituted by Sn atoms and n-type doping results, which provides electronic conductivity to ITO. ITO is extensively employed in spectro-electrochemistry as an optically transparent electrode due to its unique optical properties [28-32].

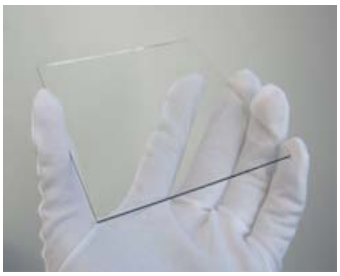


Figure 1.1: ITO coated glass

ITO electrodes have attracted the interest of many researchers due to their unique electrochemical and physical properties and wide potential window, which make them an excellent electrode substrate for constructing metal and semiconductor nano particles arrays. In several electrochemical methods the application of ITO has been reported for the detection of variety of organic compounds due to its electrical conductivity and the ease with which it can be deposited as a thin film [33-36].

Carbon electrode

Carbon surfaces are considered as an attractive materials for electrochemical analysis as they can be synthesized in different forms (from powders to fibers, foams, fabrics and composites) and are found in form of different allotropes (graphite, diamond, fullerene). Carbon-based electrodes allow scans to more negative potentials than platinum, gold or silver, as well as they have anodic potential windows. Usually carbon-based electrodes act as an inert source or sink of

electrons and therefore indirectly facilitate electro-analysis. Some important carbon based electrodes are:

a. Glassy carbon electrode

The most commonly used form of carbon electrode is the glassy carbon, which is easily available. Glassy carbon (vitreous) is a non graphitizing carbon which combines glassy and ceramic properties. It is an inert material which is gas impermeable, electrically conductive and highly resistant to chemical attack. Glassy carbon is composed of aromatic ribbon like molecules which are tangled to each other in a complicated manner.

Figure 1.2: Glassy carbon electrode

Glassy carbon is a terbostratic form of carbon which is produced by carbonizing the polymeric precursors such as copolymer resin of phenol formaldehyde or furfuryl alcohol-phenol under controlled situation of temperature (1000 – 3000^{o}C) and pressure. Theses polymers are mostly used because they provide high carbon yield on pyrolysis (ratio of carbon present after/before the carbonization is ~ 50 %). The physiochemical properties of glassy carbon are greatly affected by the starting polymer and temperature of carbonization. It is isotropic, electrically conductive and resists strong acid and alkali. Glassy carbon electrode (GCE) is suitable for electrochemical studies over wide potential range from about + 1.2 to - 0.8 V vs. SCE. GCE offers attractive electrochemical reactivity, negligible porosity (due to its low density 1.3 – 1.5 g cm^{-3}) and low background current. In addition, it is very easy to prepare and can be modified by simple methods [37-41]. Due to the specific structural orientation and excellent electrochemical properties, glassy carbon is employed as an electrode material for fabrication of sensors for monitoring of several biomolecules and drugs [42-52].

b. Pyrolytic graphite electrode

Recently, the use of pyrolytic graphite has become increasingly important in electro-analytical chemistry due to its large potential window, chemical unreactivity and ability to suppress the background current. Pyrolytic graphite is an electrode material which is highly aniosotropic and diamagnetic with the graphite monocrystal size. Due to nature of chemical bonding in graphite, two distinct planes edge plane and basal plane exhibit completely different electrocatalytic activity from each other.

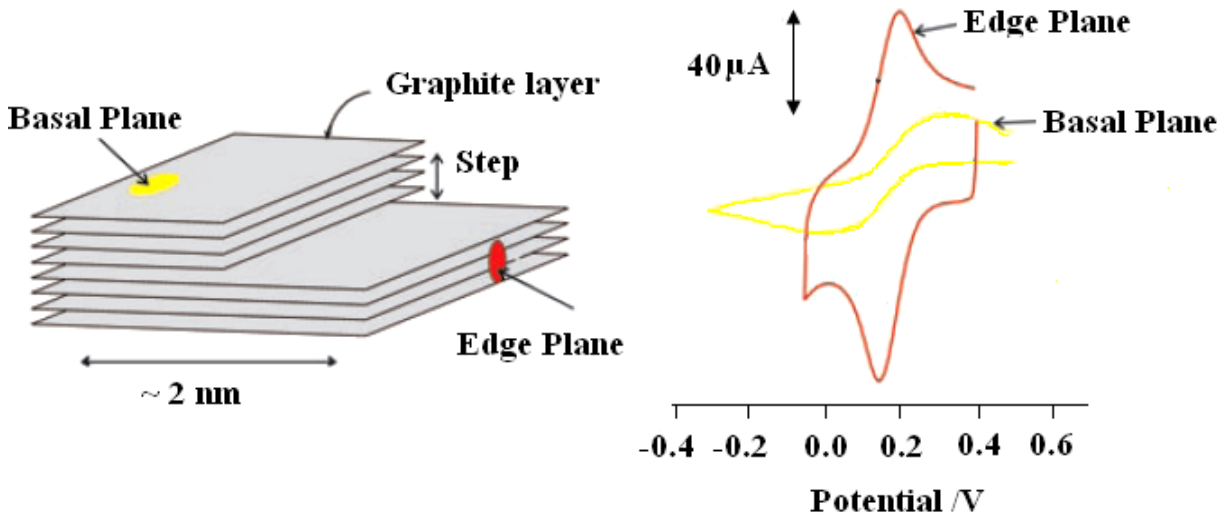


Figure 1.3: Schematic representation of basal and edge planes with step edges in HOPG, showing better CV signals at EPPG as compared to BPPG.

In highly ordered pyrolytic graphite (HOPG) electrode, the basal plane surface consists of graphite layers which arrange parallel to the surface and are separated from each other by 3.35 A^{o}, whereas the surface of edge plane consists of layers of graphite which are perpendicular to the surface. Electrode kinetics at edge plane pyrolytic graphite (EPPG) is at least three orders of magnitude faster than at basal plane pyrolytic graphite (BPPG). EPPG surface is generally rough in microscopic scale which leads to high capacitance and large surface area and is advantageous to BPPG surface [53-57]. The presence of highly reactive surface defects, which is found in the form of steps exposing the edges of graphite layers (as shown in Fig. 1.3), allow strong adsorption tendency, low detection limit, low over potential, improved signal to noise ratio and excellent

electrocatalytic activity in comparison with those obtained by the use of BPPG, glassy carbon, carbon paste and boron doped diamond electrode. Therefore, in this dissertation a wide range of important analytes have been studied using EPPG electrode, as it exhibits high degree of electrochemical properties than any of the alternative electrodes in terms of low detection limit and high sensitivity [58-68].

1.2.2 Nanomaterials for the Surface Modification

Nanomaterials exhibit unique physical and chemical properties such as increased strength, chemical reactivity and conductivity because of the effects such as quantum size effect (i.e. fluoroscene) in which the electronic properties of solids are altered with great reduction in particle size and surface effects (i.e. catalysis). In bulk materials, atoms of solids are principally located on basal plane and only relatively a small percentage of atoms are at or near the interface, but in nanomaterials many atoms (half or more) are found near the interface (edge or corners). Because of the low coordination number, edge and corner atoms are highly reactive, catalytically active and highly polarizable in comparison with the atoms on basal plane and result in some of the interesting properties of nanomaterials [69-71].

The unique and excellent physical properties of nanomaterials have placed them in the forefront of emerging technologies. Significant improvement of mechanical, optical, electrical, magnetic and structural properties is commonly observed by the application of novel nanomaterials. Advent of nanotechnology has added a new dimension to electrochemical research for the development of sensors, biosensors and electrochemical bioassays. Electrochemistry and nanotechnology are interdisciplinary fields and both are acquiring an increasing importance in the development of improved performance and reliable alternative energy devices. The interrelation between interfacial electrochemistry and nanoscience gives rise to new possibilities to construct the chemical surface by regulating the surface structure at molecular level. Rapid developments in nano particles preparation, surface modification and assembly have led to the widespread use of nanoscience in the field of electrochemistry. Nanomaterials have been widely employed to immobilize enzymes, antigens and nucleic acids on electrochemical sensor surface. These nano structured electrochemical sensors, biosensors and immunosensors have been applied in medicine (for diagnostic and detection of various severe diseases), in environmental monitoring (pollutant, pesticides, herbicides and genotoxic molecules detection) and in food quality control (micro-

organism and food toxin quantification). The fabrication and effective use of nanomaterials to modify the electrode surface presents formidable challenges and open a new range of possibilities for the construction of functional and miniaturized electrochemical devices. Unique physical, chemical and electrocatalyic properties of nanomaterials have led to the development of electrochemical sensors, which exhibit high sensitivity and selectivity. Nanomaterials possess high electrical and thermal conductivity and excellent mechanical properties due to the minimum defects in their structure. Nanomaterials are used either as modifiers of the electrochemical sensors or as the labels to enhance the electrochemical signal and can be employed as a promising material for the creation of useful electrochemical sensors and biosensors. Sensitivity and signal to noise ratio of many electrochemical sensors are significantly improved by using nanomaterials [72-75]. Nanomaterials can be prepared from carbon or polymeric species, metals and semiconductors in various nanostructures such as nanotubes, nanofibers, nanorods, nanoparticles, thin polymeric films, nanocomposites and nanowires etc. and widely employed for the investigation of large group of biomolecules and drugs due to their ability as electrode modification materials to enhance the efficiency of electrochemical sensors. Unique properties of different types of nanomaterials provide them novel catalytic, thermal, electrical and magnetic properties due to which they are being applied in commercial, medical, military and environmental sectors.

There are several types of intentionally produced nanomaterials which can be organized into four types:

(1) Carbon based nanomaterials

(2) Metal based nanomaterials

(3) Dendrimers and

(4) Composites

In following paragraphs, we are presenting brief outlines of some important carbon based nanomaterials.

Carbon based Nanomaterials

Carbon based nanomaterials are extensively used in various analytical applications. Today the nano carbon family includes many recently discovered “wonder materials” including zero dimensional (0D) fullerene, one dimensional (1D) carbon nanotubes, two dimensional (2D) graphene and three dimensional (3D) nano diamond. These nanomaterials most commonly are found in the form of ellipsoids, a hollow spheres or tubes. Spherical and ellipsoidal carbon

nanomaterials are termed as fullerenes and cylindrical nanomaterials as nanotubes. The physical, chemical and electronic properties of carbon based nanomaterials strongly depend on carbon's structural conformation and hence its hybridization states. It is stated that the small energy gap between 2s and 2p permits the promotion of s-electron to higher energy p-orbital in ground state and this promotion allows the carbon atom to hybridize into sp, sp^2 or sp^3 configuration based on neighboring atom [76-79]. Due to their unique properties carbon based nanomaterials play an important role in many areas of research including material science, biomedicine and analytical electrochemistry. Excellent chemical stability, high surface area, large potential window, electrocatalytic activity for variety of redox reactions and compatibility with biological processes make them very useful in sensing applications. Carbon based nanomaterials facilitate the electron transfer between electrode and analyte, thus increasing the efficiency of electrochemical reactions. Carbon based nanomaterials can be readily applied for the modification of electrochemical sensors to monitor numerous organic compounds, as they provide higher sensitivity, lower detection limit and faster electron transfer kinetics than other electrode surface modifiers [80-82]. Some of the frequently used carbon based nanomaterials are as follows:

Fullerene (C_{60})

Fullerene C_{60} so called as buckminster fullerene or buckyballs is one of the allotropes of carbon which can be produced by inserting large amount of current or arc in between graphite sheets in low pressure helium or argon atmosphere. Fullerene is composed of thermodynamically stable carbon shell ~ 1nm in diameter, consisting of 20 six membered ring and 12 five membered ring and is having very low density due to their shape like a hollow ball. Fullerene is nano sized group of carbon with large surface area, high reactivity and hydrophobic surface that increase their adsorption capacity towards organic molecules [83]. Fullerene is highly resistant to oxidation but shows high electron affinity and upto 6 electrons can be accommodated in lowest unoccupied molecular orbital (LUMO) of fullerene. It is known that C_{60} becomes conductive on reduction and doping with alkali metal cations. These partially reduced fullerene film can be used as an efficient electron promoter substrate for study of several redox species. C_{60} has several redox levels as well as very low solubility in aqueous solution, due to which fullerene film coated electrode can be used as mediator in electrochemical reactions of several organic compounds [84-88].

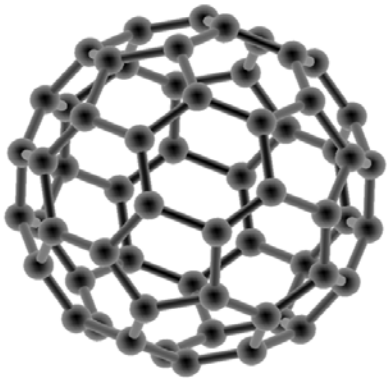


Figure 1.4: Soccer ball type structure of fullerene

Graphene

Graphene is an allotrope of carbon which is basically a one atom thick layer of graphite. It is composed of sp^2-hybridized carbon atoms which are arranged in two dimensional hexagonal shape. Each carbon atom is bonded to three other carbon atoms by the covalent bond. Graphene considered as “rising star”, has attracted an appreciable interest in many applications such as electronics, energy storage and conversion due to its high mechanical strength, high surface area and excellent thermal and electrical conductivity [89].

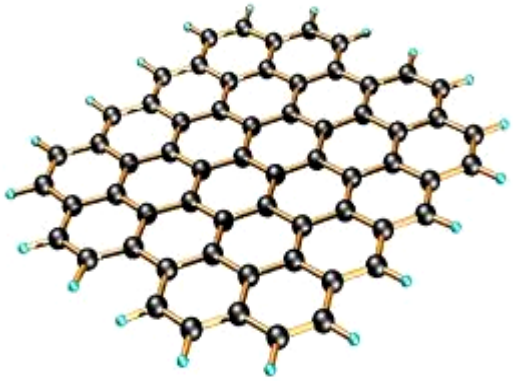


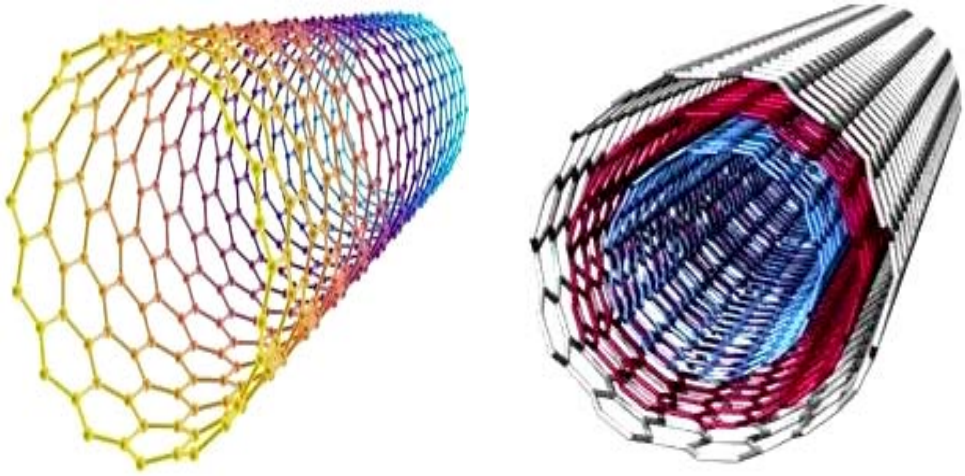
Figure 1.5: Graphene structure

Its biocompatibility permits the immobilization of enzyme and high conductivity promotes the electron transfer between electrode and enzyme. As a novel carbon based nanomaterials,

graphene and graphene derivatives have displayed excellent electrochemical properties for the detection of direct electrochemistry of enzymes, for electrochemical detection of biomolecules, drugs and heavy metal ions [90-93].

Carbon nanotubes

The most important class of carbon based nanomaterials is represented by the carbon nanotubes (CNT). Since the discovery of carbon nanotubes by Sumio Iijima in 1993, CNT have been the subject of significant interest in a magnitude of disciplines within physical sciences. CNT are the allotropes of carbon having cylindrical nano structure. CNT typically have diameters ranging >1 nm upto 50 nm with high aspect ratio (length to diameter ratio), which is significantly larger than any other material. The chemical bonding of carbon nanotubes is composed entirely of sp^2 bonds, similar to those of graphite. The CNT can be considered as rolled up graphene sheets which are rolled at specific chiral angle, deciding the properties of CNT.



(a) **(b)**

Figure 1.6: Structures of (a) SWCNT and (b) MWCNT

CNT are strongest and stiffest materials which result from the covalent sp^2 bonds formed between individual atoms. Nanotubes are categorized into single-walled carbon nanotubes (SWCNT) and multi-walled carbon nanotubes (MWCNT). The structure of SWCNT can be

viewed as molecular wires with every atom on surface, have the diameters between 0.4 and 2 nm and tube length which is many millions of times longer than diameter. MWCNT comprise of an array of multiple rolled layers (concentric tubes, 0.34 nm apart) of graphene which are held together by Van der Waals forces, where the final MWCNT have the diameter 2–100 nm [94, 95].

CNT display metallic, semiconducting or superconducting electron transport properties depending on diameter and degree of chirality, which make them very attractive for the construction of electrochemical sensors. The chirality is related to the angle at which the graphene sheets are rolled, hence the alignment of π-orbitals. CNT are synthesized usually by three main techniques; arc discharge, laser ablation and carbon vapor deposition. By altering the conditions either SWCNT or MWCNT can be synthesized. They are found suitable for the electrodes modification due to their large surface area, high electrical conductivity and better chemical and electrochemical stabilities. CNT are used in electrochemical sensors for signal amplification. Their use has the advantage of large surface area, due to which a number of biomolecules can be immobilized on them, which incresaes the number of binding sites for the detection of biomolecules and drugs. CNT based sensors offer higher sensitivity, lower limit of detection and faster electron transfer kinetics than any other conventional carbon electrode [96, 97].

It is found that carbon atoms of CNT at side wall and at the end of the tubes show different electrochemical behavior from each other. The electrochemical behavior of carbon atoms at the side walls and end of the tubes can be compared with the basal and edge planes of HOPG. The side walls of CNT are suggested to have electrochemical reactivity similar to those of the basal plane of HOPG, while their open ends show a behavior similar with edge plane of HOPG. This behavior was explained by Compton *et al.* in the electrochemical study of ferricynide at the C_{60} and carbon nanotubes modified electrode. These results were compared with basal and edge planes of HOPG. The electron transfer rate constant observed was similar for nanotubes modified electrode and edge plane pyrolytic graphite electrodes, while the fullerene (doesn't contain any open end) modified electrode resembles with basal plane of HOPG [98]. The electroactive ends of the nanotubes are easily accessible to species in solution. The electrochemical reactivity of open ends of nanotubes is observed due to the presence of edge plane defects at end of tubes, which are electroactive sites [99, 100]. Furthermore, electrocatalytic activity of CNT modified electrode strongly depends on the way of production of CNT, either by arc discharge or by chemical vapor deposition (CVD). Arc method creates CNT with closed ends (fullerene like) and chemical vapor deposition produces

CNT with open ends. The difference in electrochemical behavior is attributed to the small fraction of edge plane defects at arc-produced CNT and high density of these defects at CVD-produced CNT [101].

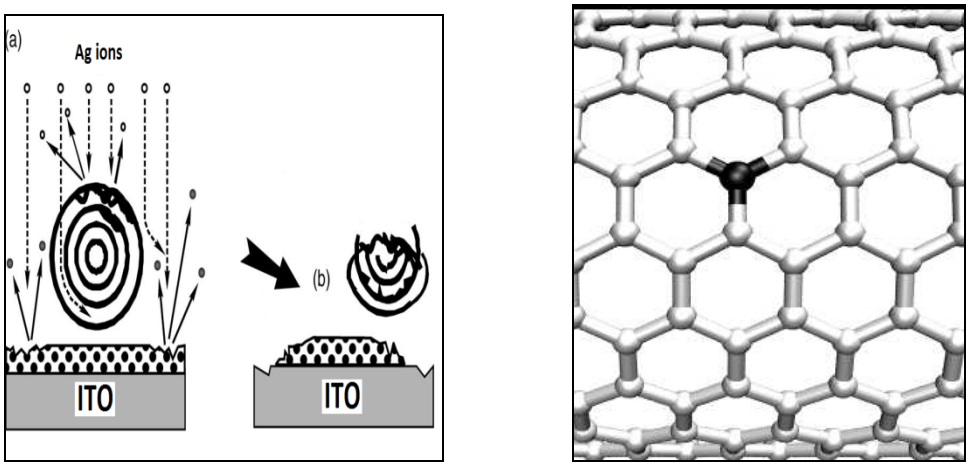
CNT are commonly synthesized by the addition of small amount of transition metals as catalysts such as Fe, Co and Ni. These metal particles are embedded in capsule of several graphene sheets due to their adhesion, which can't be easily removed. It is assumed that in normal sample of 1 g of CNT, 0.05 g metal impurities are found. Banks *et al.* reported that electrocatalysis at carbon nanotubes modified electrodes is likely due to important role of metallic impurities present in CNT. The electrocatalysis has been shown to be most likely due to Fe_2O_3 impurities [102]. Similarly Pumera and coworkers demonstrated that multi-component metallic residuals (Co, Mo and Fe) are found to be responsible for electrocatlytic oxidation of hydrazine on carbon nanotubes [103]. On the basis of above observations, it is revealed that the presence of topological edge plane defects and metallic impurities in CNT structure make them a promising candidate in constructing the modified electrochemical sensors, as a result of which they can be successfully applied for studying the voltammetric behavior of several biomolecules and drugs.

1.2.3 Effect of Heavy Ion Irradiation of nanotubes

Swift heavy ion irradiation (SHI) with energetic particles has some advantageous effects on nanomaterials, e.g. ion irradiation of nanotubes is routinely used in semiconductor industries to introduce dopant atom into nanomaterials structure in order to modify their electrical properties. In most of the studies tailoring of nanotubes properties with ion irradiation have been considered. As carbon nanotubes are highly conductive, the changes induced by irradiation are considered to be affected by knock-on atom displacement [104, 105].

Irradiation of carbon nanotubes by heavy ions such as Ag, Ni or Si leads to the formation of ion tracks (columnar defects) within nanotubes thin film due to the excitation or ionization of carbon atoms. Moreover, heavy ion irradiation leads to sputtering and creates vacancies on side walls and interstitial atoms due to which rough surface is formed which is key aspect of increasing the surface area after irradiation [106, 107]. Although, heavy ions with high energy can damage the nano structure and decrease the stability of nanotubes, however, optimizing the experimental conditions such as energy of ions, fluence of ions (ions/cm^2) and selection of heavy ions according to the thickness of film can lead to significant changes in nanotubes and then this phenomenon can

be readily applied for several analysis. The detailed application of this procedure in electroanalysis of biomolecules has been explained in chapter 3, comprising all the aspects related to heavy ion irradiation.



(a) **(b)**

Figure 1.7: (a) Ion irradiation of carbon nanotubes results in sputtering of atoms (b) Nanotubes after irradiation (black atom is dopant and white are carbon)

1.2.4 Modified Electrodes

An increased attention has been paid for the modification of electrodes using nanomaterials, due to their excellent structural, mechanical and electrical properties. Since the discovery of nanomaterials, many papers have reported the use of nanomaterials as electrode modifier. Most of the work concerns with the use of gold nano particles, fullerene and carbon nanotubes for the surface modification, demonstrating the particular structure and unique properties of nanomaterials for designing electrochemical sensors and biosensors.

AuNPs modified electrodes

In recent years, several research papers have been published using AuNPs for electrochemical applications. AuNPs modified carbon ionic liquid electrode was used for the investigation of a flavinoid; Rutin which serves as antitumor, antioxidant and anti-inflammatory agent. Modified electrode showed the increase of peak current and decrease of peak potential

which are attributed to the catalytic activity of gold nano particles [108]. AuNPs/TiO_2 composite modified indium tin oxide electrode was constructed by Wang *et al*. to study the electrochemical behavior of catechol in tea samples which showed satisfactory results with detection limit of 5×10^{-8} mol L^{-1} [109]. AuNPs have been widely applied in the study of direct electron transfer (DET) of some redox proteins. For instance, Li and coworkers reported DET immobilized hemoglobin and surfactant protected AuNPs modified glassy carbon electrode. The hemoglobin immobilized on colloidal AuNPs showed a quasi reversible redox couple at about - 0.256 V and - 0.206 V in phosphate buffer [110]. Another application of DET was reported by Frasca *et al*. in which they showed direct electron transfer of human sulfite oxidase enzyme immobilized on AuNPs modified electrode. These nano particles were covalently bonded to acid treated gold electrode where this enzyme was absorbed and an increased interfacial electron transfer and electro-catalysis was obtained [111]. A large number of biomolecules have been studied using AuNPs as enhancing materials, such as glucose [112, 113], dopamine and serotonin [114], norepeinephrine [115], epinephrine [116] and ascorbic acid [117].

Fullerene modified electrodes

Fullerene has emerged as an important electrode material due to its good sensitivity and selectivity for the detection of target analyte. Our research group developed a method for the determination of dopamine and ascorbic acid by the partial reduction of C_{60} on gold electrode. The modified electrode not only showed the electrocatalytic activity towards the analysis of dopamine and ascorbic acid, but also resolved the problem of overlapping and two distinct peaks of both analytes were observed. Therefore, it became possible to detect dopamine in presence of ascorbic acid [118]. Jahangir *et al*. proposed an approach for the investigation of endocrine disruptors which are environment pollutant, using Fullerene C_{60} modified glassy carbon electrode. The fabricated sensor exhibited a lowering in peak potential and significant increment in peak current. They used bulk electrolysis for the treatment of waste water which could lead to the path of green technology [119]. Redox process of fullerene peapod modified electrodes was checked by Sun and coworkers. Fullerene peapods are functionalized SWCNT, i.e. SWCNT are used as nano capillaries to include fullerene molecules. This work paved the way for exploring the other fullerene peapods and might be applicable for electrochemical sensing of several compounds with the retention of redox properties of fullerene [120]. Furthermore, electrocatalytic activities of fullerene modified electrodes were checked for the simultaneous detection of adenine and guanine [121] and

methionine [122]. Modified electrode exhibited high catalytic activity towards these biomolecules with excellent stability and reproducibility. The application of fullerene C_{60} modified surfaces in analysis of biomolecules are also compiled in a book chapter [123].

CNT modified electrodes

Currently, much attention has been focused on developing carbon nanotubes based nanomaterials, which are being employed for signal amplification in electrochemical sensors. Construction of efficient CNT modified electrochemical sensors is a very promising tool in promoting the electron transfer reactions of biologically important biomolecules and various pharmaceutical drugs. The first utilization of CNT in electrochemistry was reported in 1996 by Britto *et al.* [124], who used a paste of nanotubes with bromoform as binder, filled into a glass tube to study the redox reaction of dopamine. At nanotubes modified electrode the oxidation of dopamine occurred at low potential with a faster rate than found using other catalytic surfaces. Britto's pioneering study explained some unique features of carbon nanotubes and spawned several other methods for fabrication of sensors using these nanotubes.

Recently, Huang and coworkers used MWCNT modified glassy carbon electrode for the investigation of haloperidol and hydroxyzine. Both the drugs are used in the treatment of schizophrenia. The parasitic absorption of species was noticed and it was suggested that before every scan electrode must be renewed by repetitive voltammetric cycles in background electrolyte until stable response is observed [125]. The electrochemical response of hydrogen peroxide was evaluated on nafion coated CNT modified glassy carbon electrode. The perfluorinated polymer nafion was used to solublize the CNT. The modified electrode showed the oxidation of H_2O_2 at 0.2 V versus Ag/AgCl, whereas bare glassy carbon electrode did not show any peak upto 1.0 V [126].

CNT modified electrodes have been successfully applied for determining important biomolecules such as epinephrine, dopamine, norepinephrie, uric acid and ascorbic acid. Yin *et al.* developed a polymer composite MWCNT sensor for the analysis of dopamine. The authors used β-cyclodextrine-incorporated MWCNT on a polyaniline modified GCE, which showed excellent selectivity, sensitivity, stability and reproducibility in the determination of dopamine [127]. Li *et al.* observed that polypyrrole-SWCNT composite film can be used to detect dopamine, ascorbic acid and uric acid simultaneously. This film was also used for anodic determination of nitrite and showed catalytic activity towards the electrochemical response of nitrite [128]. The application of

conductive composite film containing functionalized MWCNT with poly neutral red has been reported by Yogeswaran and Chen for the simultaneous determination of mixture of biologically important compounds such as ascorbic acid, dopamine and uric acid in presence of phosphate buffer of pH 4.0. The reason of well resolved oxidation peaks was assigned to the hydrophobic and electrostatic interactions between three analytes and positively charged polymer backbone and negatively charged functionalized nanotubes [129]. Epinehrine is an important catecholamine neurotransmitter and coexists with ascorbic acid, uric acid and dopamine in biological fluids, which may interfere during the electrochemical determination of epinephrine at unmodified electrodes. Valentini *et al.* developed modified stainless steel microelectrode (diameter 300 µm) using functionalized SWCNT by electrophoretical deposition process. The presence of electron donating groups (-OH group) on SWCNT repels ascorbic acid and uric acid, while it attracts epinephrine which was electrochemically measured with detection limit of 2 µM [130].

Several drugs have been investigated using efficient carbon nanotubes modified sensors due to the unique structural and electrochemical characteristics of nanotubes. Xiao *et al.* determined an antibacterial drug chloramphenicol in milk samples. In this method they used composites of SWCNT, AuNPs and an ionic liquid (i.e. 1-octyl-3-methylimidazolium hexafluorophosphate) to modify glassy carbon electrode. The composition of the developed film and experimental conditions played beneficial role towards voltammetric response of this drug and detection limit was observed as 5 nM [131]. Another composite film of Pt nano clusters and MWCNT was used to fabricate GCE for the detection of some essential estrogens, e.g. estrone, estradiol and estriol in the real samples of blood serum. The electrode was stable and showed linear response in square wave voltammetric study over the concentration range between 2 – 50 µM, 0.5 – 15 µM and 1.0 – 75 µM for estrone, estradiol and estriol, respectively [132]. Carbon paste electrodes have been used in various electrochemical applications due to their ease of preparation, ease of cleaning the surface for renewability and compatibility with numerous types of modifiers. A MWCNT modified carbon paste electrode was used for electro catalytic oxidation of bergenin using differential pulse voltammetry and cyclic voltammetry. Bergenin is used for the treatment of gastrointestinal diseases and has anti-inflammatory, anti-HIV and anti arrhythmic activity. The modified electrode showed a considerable enhancement in kinetics of electro-oxidation of bergenin. There was no loss in electro activity of electrode for the continuous cycle sweep for 6 h and no deterioration even after 3 weeks [133]. Functionalization of carbon nanotubes

has been gaining an importance for last few years. Karadas and coworkers developed an assay based on the application of silver nano particles and - COOH group functionalized MWCNT modified GCE for the determination of an indole derivative drug, zolmitripan. This drug is used against acute migraine attacks. Under optimum experimental conditions, the modified electrode showed an enhancement in peak current of zolmitripan and limit of detection was 1.47 nM. The method was also used for the determination of this drug in tablets and human urine samples and the satisfactory results were obtained [134]. Recently, Goyal *et al.* constructed efficient electrochemical sensors based on surface modification by carbon nanotubes for studying various drugs such as norfloxacin, mometsone furroate and halobetsol propionate in pharmaceutical preparations and biological samples. An antibiotic, norfloxacin was detected using MWCNT modified EPPGE by square wave voltammetry. The modified electrode showed electrocatalytic performance towards the reduction of norfloxacin in the concentration range between 1.2 – 1000 µM and detection limit observed was 40.6 nM. The effect of norfloxacin on the catabolism of caffeine was also studied by determining its concentration in urine samples [135]. The effect of cationic surfactant, cetyl trimethyl ammonium bromide on electrochemical response of a corticosteroid; mometsone furroate was studied using SWCNT modified EPPGE. The reduction site in mometasone furroate was established by characterization of the product of reduction by ^{1}H NMR and FT-IR measurements [136]. Another topical corticosteroid halobetasol propionate was detected using SWCNT modified electrode in pharmaceutical preparations by square wave voltammetry and cyclic voltammetry in phosphate buffer of pH 7.20. Modified electrode exhibited enhancement in peak current and lowering in peak potential as compared to bare electrode, demonstrating the elctrocatalytic ability of SWCNT towards the reduction of this drug [137]. Fan *et al.* used an ionic liquid (1-butyl-3-methylimidazolium hexafluophosphate) -SWCNT coated glassy carbon electrode for voltammetric determination of methylparathion. The electrode showed good electro-catalytic behavior towards methylparathion and also for its hydrolysate (*p*-nitrophenol) [138]. Analytical sensing by the application of CNT modified electrodes is found to result in low detection limit, high sensitivity and selectivity, excellent reproducibility and stability, reduction of over-potential and resistance over surface fouling. These excellent properties of CNT make them fascinating material for the development of outstanding electrochemical sensors and the continuous increasing research interest in this field firms the belief that CNT based electrochemical sensors may lead to dramatic changes to future sensors.

1.3 METHODOLOGY OF PRESENT INVESTIGATION

Voltammetry is a utilization of potential ramp with the subsequent measurement of current observed from the reaction of any chemical species occurring at electrode. The physico-chemical phenomena originating at electrode-solution interface has led to development of a new range of voltammetric techniques. A brief description about the methodology employed in present electrochemical investigation comprising theoretical aspects of these techniques is being presented in forthcoming sections:

1.3.1 Voltammetric cell

Unlike potentiometry measurement, which employs only two electrodes, voltammetric measurement utilizes a three electrode electrochemical cell (working, auxiliary and reference) to minimize the ohmic resistance. The working electrode makes contact with the analyte and applies the desired potential in a controlled way followed by facilitating the transfer of charge to and from the analyte. The reference electrode, whose potential is constant and can be taken as standard, against which the potential of other electrodes can be measured. Commonly used reference electrodes are silver-silver chloride electrode (Ag/AgCl, E^o = 0.222V) or the saturated calomel electrode (SCE, Hg/HgCl, E^o = 0.244 V).

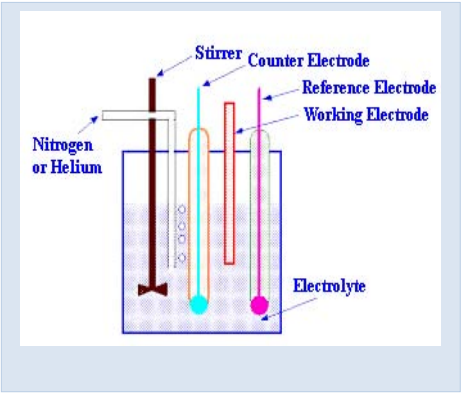


Figure 1.8: Voltammetric Cell

The counter electrode or auxiliary electrode serves as a source or sinks for electrons and passes the current needed to balance the current observed at the working electrode. The current flows

between working and auxiliary electrode due to oxidation or reduction of measuring material. This current is recorded as a function of potential, imposed on working electrode which is expressed with respect to that of a reference electrode. A supporting electrolyte is added to analyte solution which must not react at working electrode at potential being used and is used to reduce effect of migration and solution resistance.

Figure 1.9: Electrochemical Work Station (BAS) used in the present investigation

The voltammetric experiments are carried out with the help of a Bio analytical system (BAS) which allows the accurate application of potential function and the measurement of resultant current.

1.3.2 Voltammetric techniques

The voltammetric techniques provide a marked improvement in sensitivity, versatility, speed of analysis over the original direct current polarography and are in growing demand for the analysis of metal speciation and of pollutant and their metabolites, clinical and drug analysis [139]. Different voltammetric techniques are distinguished from each other primarily by the applied potential that is used to facilitate the determination process at working electrode. A short discussion of the voltammetric techniques, which have been employed to perform analysis, is being mentioned here:

1. Cyclic Voltammetry

Cyclic voltammetry (CV) is an important modern electroanalytical method which is used for qualitative analysis of redox process, for understanding reaction intermediates in oxidation-reduction reactions, evaluation of electron transfer kinetics and the reversibility of a reaction. This technique is capable for rapidly determining the redox behavior over a wide range of potential. The CV has been used by several researchers for monitoring of various biomolecules. Fast CV was used to monitor endogenous noradrenaline release in rat brain slice at carbon fiber micro electrode [140]. The analysis of dopamine/serotonin response to acute Escitalopram was carried using CV [141]. The CV is based on varying the applied potential at working electrode in both forward and reverse direction (at some scan rate) while measuring the current. The working electrode is directed to triangular potential sweep, whereby, the potential rises from a start value E_i to a final value E_f and then returns back to the start potential at a constant potential sweep rate. Sweep rate applied can vary from few milivolts per seconds to hundreds of volts per seconds.

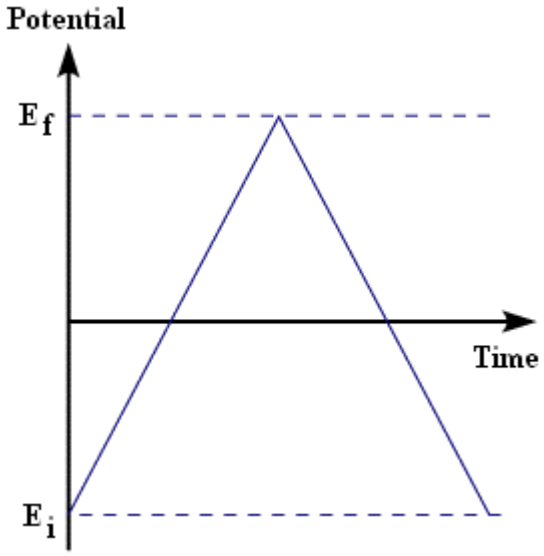


Figure 1.10: A triangular potential waveform in CV

The current measured during these experiments is termed as current density, which is plotted against the applied potential and the resultant curve is termed as cyclic voltammogram. A

peak observed in CV at definite potential is characteristic of a reaction occurring at electrode surface. A typical cyclic volatmmogram recorded for a single electron transfer reaction can be shown as follows:

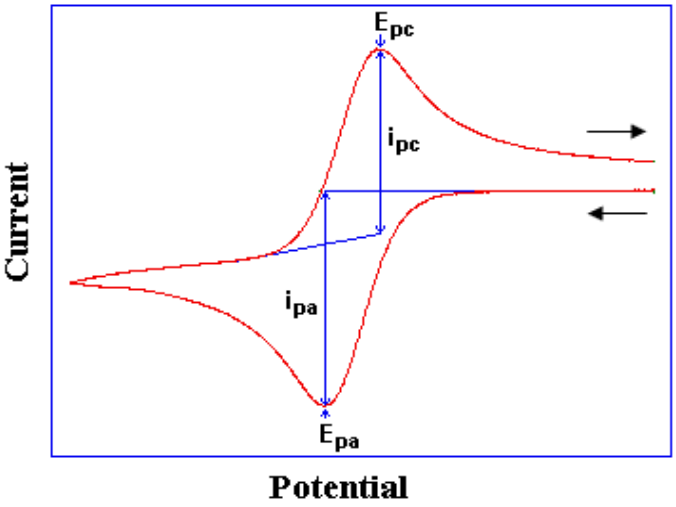


Figure 1.11: A typical cyclic voltammogram for reversible redox system

The important parameters in cyclic voltammograms are the magnitude of the peak potentials (E_{pc}, E_{pa}) and peak currents (i_{pc}, i_{pa}) of cathodic and anodic peaks, respectively. If the electron transfer rate in both forward and reverse scan is high, the reaction is defined as electrochemically reversible and the peak separation is given by:

$$\Delta E_p = [E_{pa} - E_{pc}] = 2.303\ RT / nF$$

Where, n is the number of electrons transferred, R is the universal gas constant (8.314 J mol^{-1} K^{-1}), T is the absolute temperature in Kelvin and F is the Faraday's constant (96485 C mol^{-1}). Thus, for a reversible redox reaction the value ofΔE_p should be 0.059/n V at 25^oC. Practically it is difficult to achieve this value because of the potential drop caused by resistance between working and reference electrode. This is especially a problem at high sweep rate, when current is observed as large but simultaneous resistance is also increased. Slow electron transfer at the electrode surface due to irreversibility causes the value of ΔE_p to increase and separation of peak potential is found greater than indicated by above equation. If the process is reversible at low sweep rates and becomes irreversible at higher sweep rates it is said to be quasi-reversible.

The peak current for a reversible system is explained by the *Randles-Sevcik* expression (at 25^{o}C):

$$i_p = (2.686 \times 10^5)\, n^{3/2} A\, D^{1/2}\, C_o v^{1/2}$$

where, i_p refers to the peak current (Ampere), A is the surface area of electrode (cm^2), D is the diffusion coefficient ($cm^2\, sec^{-1}$), C_o is the concentration of anlyte in mol $cm^{-3}$and v is the scan rate in Vs^{-1}. The relationship between concentration and peak is particularly very important in analytical applications and in elucidation of electrode mechanism. The value of i_{pa}/i_{pc} should be identical for a reversible couple at all scan rates.

CV has become increasingly popular in several areas of analytical chemistry since it enables a wide potential range to be scanned swiftly for reducible or oxidizable substances. This ability together with its variable time range and excellent sensitivity makes this technique the most versatile electrochemical technique [142]. Although CV is extensively used for the redox characterization of molecules and qualitative investigation of chemical reactions, however, there are some disadvantages, inherent in this technique. The effect of slow heterogeneous electron transfer on electrochemical reaction can not be ignored. The ratio of faradic peak current to charging peak current decreases with increasing v, as i_p is proportional to $v^{1/2}$ and this tendency reduces the possibility of measurements at high scan rates. Moreover, it must be mentioned that its advantages are especially in the realm of qualitative or diagnostic purposes. Quantitative measurements (rate or concentration) are best obtained via other techniques e.g. pulse techniques. Excellent sensitivity is achieved using pulse techniques which eliminate charging current by about 6.5 times [143].

2. Square Wave Voltammetry

Square wave voltammetry (SWV) is a powerful electroanalytical technique that can be used in both electrokinetic and analytical measurements of redox system [144]. This technique originates from Kalasouk commutataor and Barker's square wave polarography. SWV offers the advantages of excellent sensitivity, high speed, low detection limit and rejection of background current [145]. From literature survey it is revealed that several biolmolecules and drugs have been analyzed using this technique with great sensitivity and low detection limit. A simple and rapid voltammetric method was developed for quantitative determination of albendazole with detection

limit of 6.2 × 10^{-5} M [146]. Similarly, SWV was developed for the determination of antiproliferative and virostatic drug; azidothymidine by Vecak *et al.* which is used in treatment of human immunodeficiency virus type infection and was found as an effective tool for the analysis of this drug in cell culture and pharmacokinetics [147]. The electrochemical behavior of trimetazidine hydrochloride was investigated on glassy carbon electrode by SWV, detected this drug in concentration range 5 × 10^{-8} to 5 × 10^{-6} M with detection limit 2 × 10^{-8} M [148].

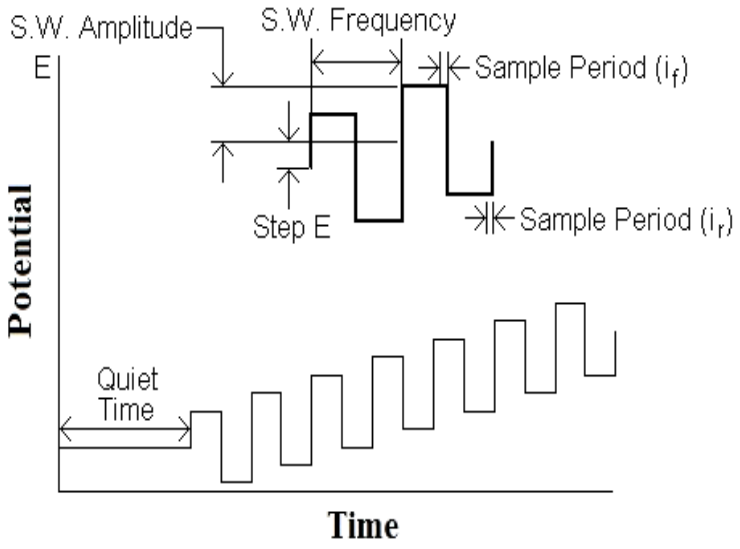


Figure 1.12: Square wave potential sweep

Square wave voltammograms can be obtained in less than 10 ms. The excitation signal in this technique is achieved by superimposing the symmetrical square wave pulse (having amplitude E_{sw}) on staircase signal of step height ΔE, where the forward pulse coincides with the staircase step. The current at the beginning of pulse is subtracted from the current at the ending of pulse so that contribution of charging current to current signal can be minimized. This is due to the charging current depends on the difference between the electrode potential and its potential of zero charge. The difference between forward current (i_{for}) and reverse current (i_{rev}) provides net current (Δi) which is proportional to the concentration of electroactive species, the potential at which the peak corresponds to $E_{1/2}$. The differential current is plotted against potential measuring the oxidation or reduction of the species as a peak. Due to the negligible contribution of charging current to the signal, detection limit can be achieved in the range in nano molar concentrations

using SWV. The sensitivity is found to increase because the net current achieved is larger than either forward or reverse component, which makes this technique more sensitive in comparison to normal pulse voltammetry and differential pulse voltammetry [149, 150]. The precision of this analysis can be increased by averaging signal data from several square wave voltammetric scans.

The ability to measure faradic current at the time when double layer charging current is negligible is primarily responsible for the success of SWV. The measurement speed coupled with signal averaging permits the experiment to be performed repetitively and increase signal to noise ratio. Such qualities of SWV make this technique very useful for the analysis of trace amount of drug compounds in their dosage forms and biological samples.

1.3.3 Controlled Potential Electrolysis

Controlled potential electrolysis is (CPE) one of the most promising electrochemical methods also known as bulk electrolysis, used for the synthesis of both organic and inorganic species, electroplating of metals and for the determination of experimental value of 'n'[151]. CPE generally employs a three electrode system, which is controlled by a potentiostat. The potentiostat automatically carry out the function of maintaining the working electrode's potential constant at any predetermined value during entire course of electrolysis. CPE is carried out in three chambers electrochemical cell. The chamber of working electrode is continuously stirred to provide maximum mass transport during electrolysis. For ideal performance, working and auxiliary electrodes should have large surface area. The fundamental concept of CPE is that if an electrode is maintained at constant potential with respect to a reference electrode, a particular electrochemical reaction is found to occur and entire current, which flows is due to this reaction. The total charge (Q) passed during the experiment is calculated by the integration of current. This charge is related to the number of electrons (n), which are transferred per molecule and number of moles of electroactive species (N) initially present. By applying Faraday's law of electrolysis:

$$Q = nFN$$

Where, F is Faraday's constant and its value is known. Hence, if one of n or N is known then other can be calculated. CPE is significantly different from that of voltammetry (in which only small amount of electroactive molecule of interest is electrolyzed). The rate of such reactions is determined by the mass transfer of material to the electrode surface not by the concentration of material. The rate can be increased by stirring the solution more rapidly or by increasing the

surface area of working or auxiliary electrode. The CPE is the most convenient method for synthetic purposes and the product obtained can be characterized by other analytical techniques including NMR, UV-Vis and FT-IR techniques [152, 153].

1.4 ANALYTES OF INTEREST

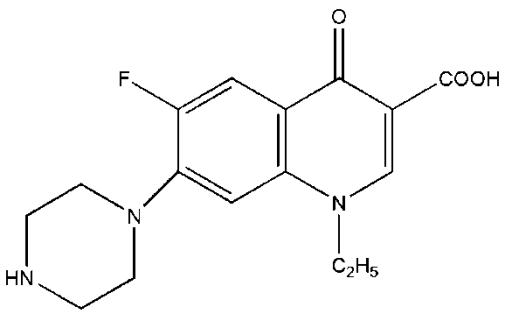
The biomolecules are essential substances for all living beings due to their active participation in several physiological processes. A minute change in their concentration may lead to several physical or mental disorders resulting in severe health problems. In last few decades the use of pharmaceutical drugs to prevent various diseases has gained much attention. It is suggested that these drugs should be prescribed in a controlled way. The overdose of these drugs may cause several side effects on human body. The misuse of drugs to enhance the performance at the site of competitive games has also become an increasing problem. With the ongoing advancement in biomedical technology, drugs have become more potent, more effective and more dangerous. Therefore, ultrasensitive and selective detection of biomolecules and pharmaceutical drugs including doping agents is highly desirable.

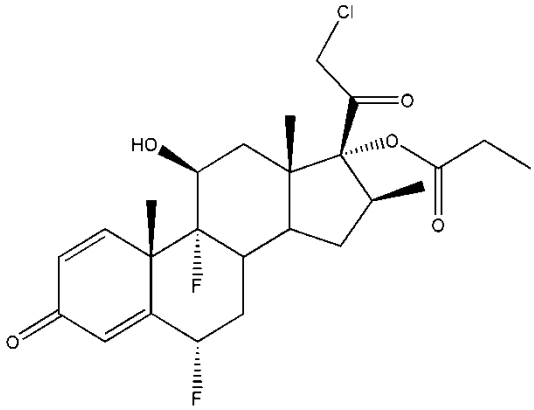
Antibiotics, also known as antibacterials, are most frequently prescribed medications that are used to destroy or kill the bacteria. Different type of antibiotics affects different bacteria in a different way. **Norfloxacin** is a fluoroquinolone antibiotic which shows broad spectrum antimicrobial activity against a wide range of bacteria. It is most commonly used to treat urinary tract infections. Its antimicrobial action results from the inhibition of bacterial DNA synthesis, thereby inhibiting cell division. Overdose of this drug may lead to several side effects such as nausea, diarrhea, dizziness, headache and stomach cramps [154]. Moreover, it is observed that prolonged administration of norfloxacin affects the catabolism of caffeine by lowering its demethylation due to which accumulation of caffeine in human body increases, leading to several health disorders [155]. The effects and side effects of norfloxacin on human health triggered our interest to detect this drug in human biological samples. Hence, we determined this drug using nanotubes modified electrode based on its electrochemical reduction and its effect on caffeine catabolism has also been elucidated.

Neurotransmitters are endogenous brain chemicals that are released from presynaptic nerve terminal of neurons and communicate the information throughout our brain and body. They can affect concentration, mood, sleep and weight and in imbalance form may cause adverse symptoms.

They are of two types; inhibitory and excitatory. **Serotonin** is an inhibitory neurotransmitter, i.e. it does not stimulate the brain but its adequate amount is necessary for stable mood. It plays an important role in emotional wellness of an individual [156]. **Epinephrine** is an excitatory neurotransmitter that is responsible for stimulating process of the brain and is secreted in situation of stress. Therefore, it is abused by athletes in sports to enhance their performance and to prepare them for facing the situation of mental pressure. It is a "doping agent" which has been banned by WADA. Both of these neurotransmitters are very important as they are used for the treatment of neurological diseases, including Parkinson's disease and Alzheimer's diseases [157]. Because of their several physiological functions, they have been determined in human urine and plasma using highly sensitive electrode. Ag ion irradiated carbon nanotubes modified electrode was used for the simultaneous determination of both neurotransmitters in this study.

Corticosteroids are man-made chemicals that closely resemble the hormones produced by adrenal cortex of vertebrates. Corticosteroids reduce the inflammation and affect the immune system. Topical corticosteroids are used for the localized treatment of skin from various inflammatory skin disorders. One of the important topical corticosteroids is H**alobetasol propionate,** which is used as antipruritic, anti-inflammatory and vasoconstrictive agent [158]. It is very effective in the treatment of plaque psoriasis and severe atopic dermatitis. It has been reported that about 1% of world population is affected by psoriasis. It is a highly potent corticosteroid that reduces the swelling, itching and redness [159, 160]. However, overdose of this drug may cause atrophy, leukoderma, acne and urticara, which make mandatory that there should be a method to determine its concentration in various biological samples. Therefore, we have detected this drug using SWCNT modified EPPGE based on its reduction and also established the site of reduction.

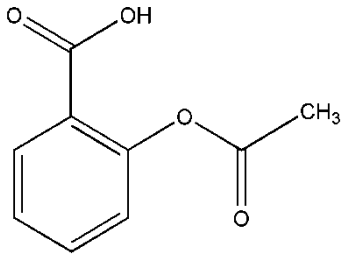


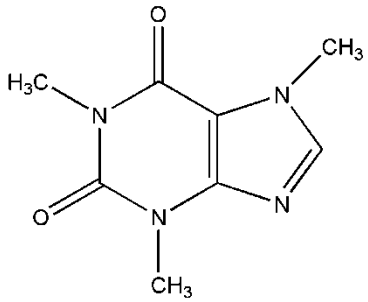


[Norfloxacin] **[Halobetasol Propionate]**

β_2-agonists are bronchodilators which cause muscle relaxation and dilate the bronchial passage making breathing easier. Asthma is a worldwide chronic disorder that results in the obstruction of intermittent airflow. **Salbutamol** is a highly selective bronchidialtor that is used in treatment of bronchial asthma and other airways obstructive disorders [161]. It can be taken either orally or using inhaler devices. There is strong evidence that salbutamol can increase the performance of healthy athletes due to which it has been listed as a "doping agent" by WADA [162]. The determination of salbutamol is of great significance because of it several uses and misuses and has been studied in this work.

The most frequently used drugs are analgesic drugs that are used to achieve "analgesia", i.e. relief from pain. Analgesic drugs also known as pain killers, are of two types: non steroidal anti-inflammatory drugs (NSAID), which alleviate pain reducing local inflammatory responses and opiod drugs that act on the brain. **Diclofenac** is a NSAID that reduces the substances in body that cause inflammation and pain [163, 164]. But its long term use is harmful to body and may cause ulcer, bleeding or holes in stomach or intestine. **Aspirin** is also a NSAID that is used to treat pain and also to reduce fever or inflammation. Aspirin is also used to prevent heart strokes and chest pains. **Caffeine** is a stimulant drug and is also used as an analgesic adjuvant in combination with certain analgesic drugs. It is the most widely used stimulant drug that can be considered as a doping substance as its high doses produce exciting effects [165-167].





[Aspirin] **[Caffeine]**

Despite the several benefits of these drugs, many disadvantages are also associated with their use. Hence, an attempt has been carried out in the present work to detect these drugs in different physiological samples using voltammetric sensors and it is believed that the development of these sensors may provide a significant contribution in the field of biomedical research.

1.5 THESIS LAYOUT

Detection of biomolecules and pharmaceutical drugs in human body fluids has always been a topic of considerable significance as it gives a big support in bio-analytical research field. Several methods have been reported for detecting these molecules. Most of the methods are based on the fact that these biomolecules and drugs are found in body in micro or nano molar range; hence, it is usually difficult to achieve a very low detection limit using electrochemical methods. However, nanomaterials modified sensors fulfill this task very effectively, which can be attributed to their high sensitivity and low over potential. Hence, in this dissertation attempts have been made to modify the surface of electrodes by the use of nano structured substances and detection of a variety of biologically important molecules and drugs has been carried out. It is believed that the modified electrochemical sensor will continue to be an important aspect of biomedical sensor development. The whole work has been systematically organized in six chapters in order to clearly present the results of investigations.

Chapter 1 Introduction

Chapter 2 MWCNT modified electrode for monitoring the effect of norfloxacin on caffeine catabolism

Chapter 3 Heavy ion irradiation of MWCNT: a study for the electrochemical determination of neurotransmitters

Chapter 4 SWCNT modified sensor for the investigation of halobetasol; a topical corticosteroid

Chapter 5 SWCNT based electrochemical sensor for salbutamol; a doping agent

Chapter 6 Determination of important analgesic drugs using bare EPPGE

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CHAPTER 2

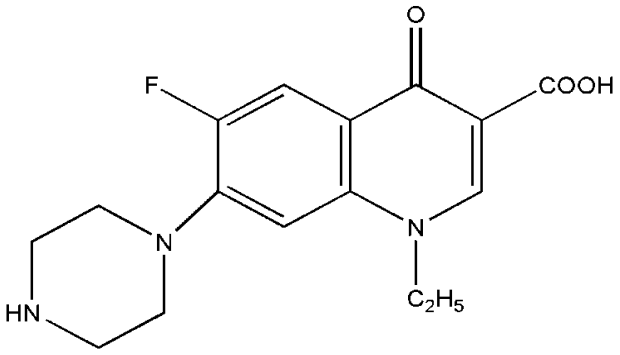
MWCNT Modified Electrode for monitoring the effect of Norfloxacin on Caffeine Catabolism

2.1 INTRODUCTION

The utilization of nanomaterials in the development of electrochemical sensors is of great importance due to their remarkable electrochemical properties such as good conductivity, large surface area and high sensitivity and selectivity. There are several nanomaterials which can be used to modify the sensor surface and have been shown to be very effective for the improvement of sensor performance. The carbon based nanomaterials have great advantages over other conventional modifiers. Among the various carbon based nanomaterials used for electrode modification, carbon nanotubes have been considered to be the most effective due to their excellent and well known electro-catalytic properties [1].

Norfloxacin (NFX) [1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolene-carboxylic acid] is a synthetic fluoroquinolone antibiotic which exhibits broad-spectrum antimicrobial activity against many pathogenic Gram-positive and Gram-negative bacteria including gentamicin-resistant *Pseudomonas aeruginosa* and methycillin-resistant *Staphylococcus aureus*. NFX is widely used in the treatment of respiratory and urinary tract infections, ocular and skin infections, gonococcal urethritis and infectious diarrhea [2]. Although, NFX is clinically important, it causes several side effects, such as, headache, depression, dizziness, nausea and vomiting [2]. The administration of NFX has also been found to affect the metabolism of caffeine through lowering its demethylation (its catabolism) process in the human system. It has also been reported earlier that the prolonged administration of NFX increases the caffeine concentration in the extra cellular fluids of the human body due to its retarted demethylation [3, 4]. Additionally, the high concentration of caffeine accumulation in the human body also reflects with numerous clinical disorder including coronary vasospasm and variety of arrhythmias [5, 6]. It is also suggested that patients with known history of arrhythmias should curtail the intake of caffeine products [7, 8].

In view of such a clinical importance of NFX and its relation with caffeine, it is desirable to develop a single step, sensitive, selective, quick and less expensive method for the determination of NFX and to monitor its effect on caffeine catabolism in the biological fluids. A number of studies have been reported for the individual determination of NFX and caffeine in biological fluids such as: HPLC [9]; spectrophotometry [10]; spectrophotometry with the aid of chemometric [11]; capillary electrophoresis [12]; spectroflourometric [13]; kinetic spectrophotometric [14] etc. However, rare attempts have been made to monitor the effect of NFX on catabolism of caffeine in biological fluids [3]. The methods reported for the determination of NFX alone or to monitor its effect on caffeine catabolim require expensive instruments along with complicated time consuming pretreatment and derivatization process.



[Norfloxacin]

Generally, electrochemical techniques have simplified the testing procedures, including home-use devices [15, 16]. Electrochemical methods based on nanomaterials modified electrodes have attracted attention in the last decade for the determination of biomolecules and drugs owing to their high electrical and optical properties [17-19]. Thus, the highly conducting characterstics of nanomaterials (e.g. MWCNT) can be utilized to develop a lable free method for the NFX determination and to monitor its effect on caffeine catabolism by exploring their direct electron transfer processes in the biological fluids. The MWCNT was depsoited on the pyrolytic graphite due to its large operational potential range and less background current [20, 21]. Thus, the present work is focussed with two objectives. First, the development of a simple and selective method for NFX detection in the urine samples using a MWCNT modified pyrolytic graphite (MPG) electrode

and second, to investigate the effect of NFX on the caffeine catabolism (or demethylation) through its electrochemical determination in urine samples. This is the first report of the selective detection of NFX in the patients urine samples based on its electrochemical reduction using a solid MPG electrode. In this work we have also monitored the caffeine catabolism (or prevention of caffeine demethylation) and its acculumation in the human urine samples.

2.2 EXPERIMENTAL

2.2.1 Instrumentation

The voltammetric studies were carried out using a computerized BAS (West Lafayette, USA; CV-50W), equipped with three electrode cell system. An unmodified pyrolytic graphite electrode (UPG) or MPG electrode was used as working, an Ag/AgCl (3M NaCl) (Model MF-2052 RB-5B) as reference and a platinum wire as an auxiliary electrode. The pyrolytic graphite pieces were obtained as a gift from Pfizer Inc., New York, USA. The field emission scanning electron microscopy (FE-SEM) instrument (JEOL-JSM 7400) was used to examine the surface morphology of the MPG electrode. High-performance liquid chromatography (HPLC) studies were carried out on Agilent 1100 series system equipped with RP-18e (5µm) column. The mobile phase was acetonitrile-water (20 : 80) and was used at the flow rate of 1 mL min^{-1}. The urine sample was filtered using a 0.5 µm membrane filter (Millipore) before injection and 5 µL was injected in HPLC. The absorbance of eluent was monitored at 260 nm. The pH of the buffer solutions was measured using digital pH meter (century India Ltd.; model CP-901). Ultrasonic machine was used to acquire well dispersed suspension of MWCNT in DMF solution.

2.2.2 Chemicals and reagents

Caffeine was obtained from Sigma-Aldrich and NFX was obtained as a gift from Ishita Drugs and Industries Ltd. Ahmedabad, India [Batch No. 0104/2010]. Both the compounds were used as received without further purification. Phosphate buffers of appropriate pH and ionic strength (1.0 M) were used. MWCNT of > 98 % purity was received from Bucky, USA. NFX containing tablets of different companies, Norflox (Okasa Pvt. Ltd., Mfg. Batch No. MV-1050), Norflox-Tz (Okasa Pvt. Ltd., Mfg. Batch No. MV-1061) and Powerflox (Cipla Ltd., Mfg. Batch

No. DV-0143) were obtained from the local market of Roorkee. All other reagents used were of analytical grade and the double distilled water was used throughout the experiment.

2.2.3 Preparation of MPG

The MPG electrode was prepared as follows. At first, the pyrolytic graphite surface was rubbed on an emery paper and then washed with double distilled water followed by softly touching it onto a tissue paper. The suspension of MWCNT was prepared by dispersing 0.5 mg of MWCNT in 1 mL of DMF. The well dispersed suspension of MWCNT in DMF was achieved by gently agitating the solution mixture for one hour in an ultrasonic bath. The optimized amount of MWCNT was casted onto the UPG surface and then electrode surface was dried at room temperature for 6 h.

2.2.4 Analytical procedure

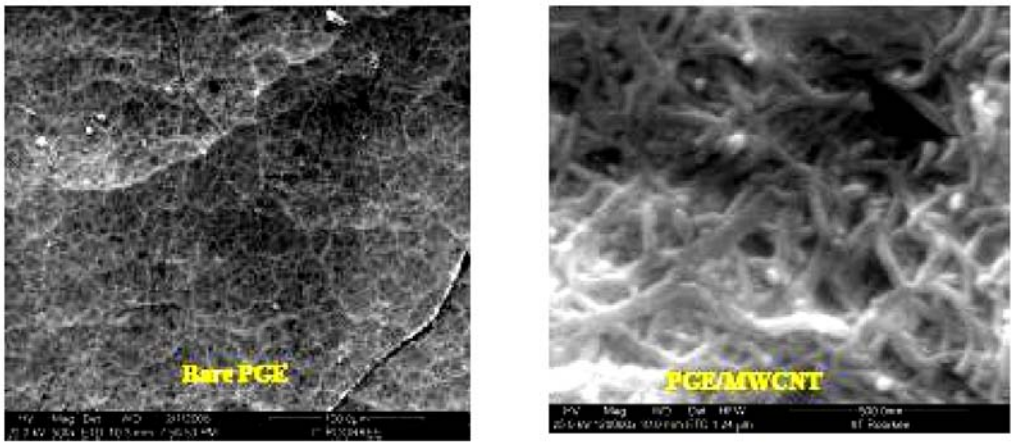
NFX is partially soluble in water but completely soluble in acidic media. Therefore, to preapre the stock solution (1mM) of NFX, the required amount of NFX was dissolved in 0.5 mL of HCl (0.1 N) and double distilled water. The NFX solutions for the voltammetric experiments were prepared by adding the required volume of the stock solution to the phosphate buffer. The solution was deoxygenated by bubbling high purity nitrogen for 20 – 30 min., before recording the voltammogram. As NFX strongly adsorbs at the MPG electrode, the surface was regenerated by the application of 0.1 V potential for 60 s after each run to remove the adsorbed material. The stock solution of caffeine (2 mM) was prepared by dissolving its required amount in the double distilled water. The urine samples of patients taking caffeine (200 mg, two dose a day) and undergoing treatment with NFX (400 mg, twice daily) were obtained every day. The morning first urine of the patients was collected for 5 days from the Institute Hospital after the permission from ethical clearance committee of the IIT-Roorkee. The AC impedance spectra (charge transfer resistance (R_{ct}) were recorded using a EG&G PAR 273A potentiostat/galvanostat and a lock-in amplifier (PAR EG&G, Model 5210), linked to a personal computer.

2.3 RESULTS AND DISCUSSION

2.3.1 Surface characterization

At first, the surface of UPG and MPG electrodes was examined by the scanning electron microscopy (SEM). The results show a well dispersed MWCNT at the MPG electrode surface as

shown in **Fig. 2.1**. In order to confirm the effectiveness of surface modification procedure, surface area of UPG and MPG electrode was calculated. For this purpose, cyclic voltammograms (CVs) of 1 mM $K_3Fe(CN)_6$ at different scan rates in 0.1 M KCl as supporting electrolyte, were recorded at UPG and MPG electrodes. A redox couple was noticed due to the Fe^{+3}/Fe^{+2} at both the surfaces, however, a significant increment in peak current at MPG electrode was observed as compared to the UPG electrode and the peak separation between the redox couple decreased to 70 mV. The surface area was calculated from the slopes of the i_p *vs.* $v^{1/2}$ plots and found to be 0.0744 and 0.2153 cm^2 for the UPG and MPG electrodes, respectively. These results clearly indicate the formation of highly conducting MPG electrode with ~ 2.8 fold larger surface area than the UPG electrode. The modified electrode was also characterized by electrochemical impedance spectroscopy by obtaining the Nyquist plots. The frequency was scanned from 0.1 Hz to 1 MHz at the open circuit voltage with the acquisition of five points per decade in the solution containing 5 mM $[Fe(CN)_6]^{3-/4-}$.



(a) **(b)**

Figure 2.1: SEM images of bare PGE (a) and MWCNT/PGE (b).

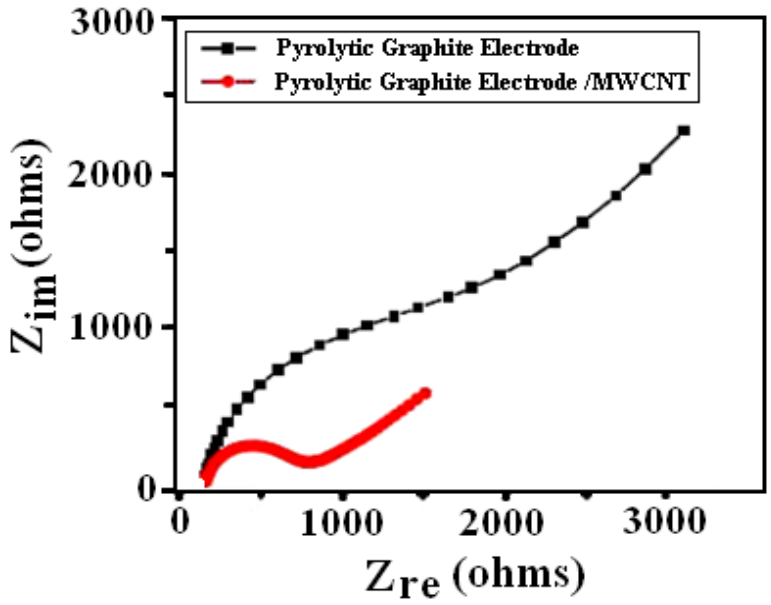


Figure 2.2: Nyquist plots of bare PGE and MWCNT/PGE in 5 mM $[Fe(CN)_6]^{3-/4-}$.

Fig. 2.2 shows the Nyquist plots obtained for bare pyrolytic graphite electrode and MWCNT modified pyrolytic graphite electrode. For the bare pyrolytic graphite electrode, the plot showed a semicircle (black line), the R_{ct} was about 3500 Ω, however, the R_{ct} value for the MWCNT modified pyrolytic graphite electrode significantly decreased to 794 Ω. The decrease in the R_{ct} for the MWCNT modified pyrolytic graphite electrode clearly indicates the ability of MWCNT to promote the electron-transfer reactions at the electrode surface.

2.3.2 Electrochemical behavior of NFX at MPG electrode

CVs were recorded for 400 µM NFX at UPG and MPG electrode using a sweep rate of 50 mV s^{-1}. The NFX is irreversibly reduced giving rise to a small peak at - 1385 mV at UPG electrode as shown in **Fig. 2.3**. It can be seen that an improved response is observed at MPG electrode as compared to UPG electrode with increase in peak current at the E_p of - 1315 mV. This behaviour suggests that MWCNT acts as an efficient electron promoter to enhance the rate of NFX electrochemical reduction. No peak was found in the reverse scan at UPG and MPG electrodes confirming that reduction of NFX occurs in an irreversible electrode reaction. CVs of 400 µM NFX were recorded by varying the scan rates ranging from 10 – 500 mV s^{-1} at MPG electrode. The nature of i_p *vs.* v plot shows that the reduction of NFX at the MPG electrode is governed by the

adsorption process [22] as shown in the inset of Fig. 2.3. The dependence of peak current on scan rate is expressed by the equation:

i_p (μA) = 0.624 v + 2.564

with the correlation coefficient of 0.994, where v is scan rate (mV s^{-1}). Since SWV is one of the widely used techniques due to its higher sensitivity and lower limit of detection, detailed studies are carried out using SWV. The square wave voltammograms (SWVs) of 300 μM NFX were recorded at MPG electrode in phospate buffer (μ = 0.1 M) as shown in **Fig. 2.4**. At UPG, NFX was reduced at - 1355 mV with a low peak current value, whereas, at MPG electrode the reduction peak was observed at - 1285 mV with a significant increment in the peak current value. Significant improvement of peak current together with a decrement in peak potential (~ 70 mV) clearly indicates that MWCNT catalyzes the reduction of NFX due to their unique properties, such as, increased surface area, high electrical conductivity and embedded metals present in their cavities [23].

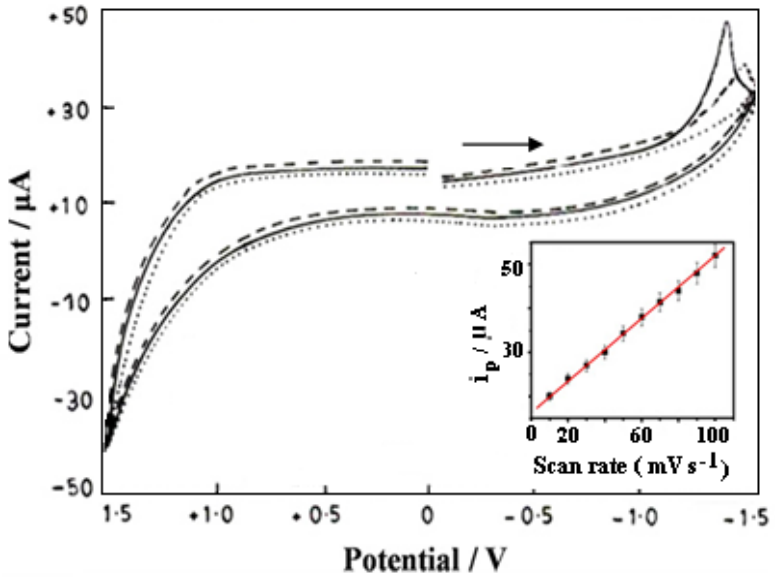


Figure 2.3: Cyclic voltammograms observed for 400 μM NFX at 50 mV s^{-1} at UPG (---) electrode and MPG electrode (—) in pH 2.1. The background at MPG is shown as (…). Inset shows the effect of scan rate on peak current of NFX.

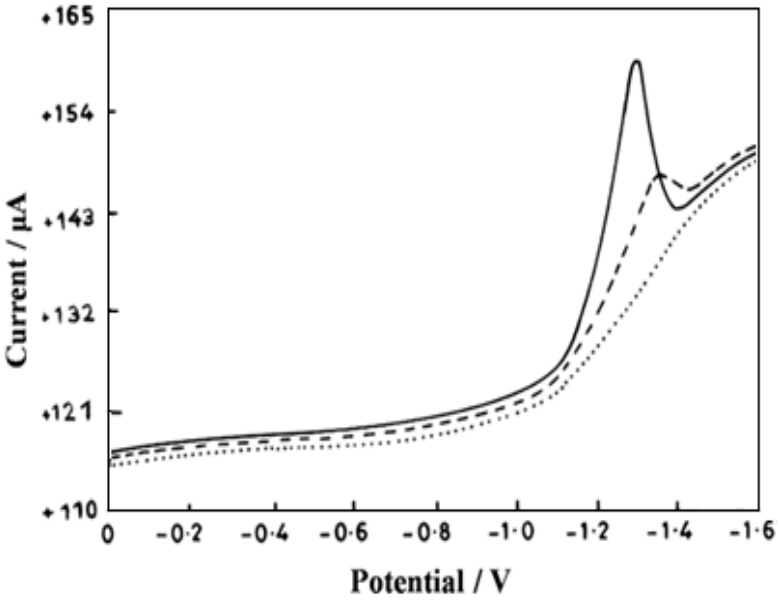


Figure 2.4: Square wave voltammograms observed for 300 µM NFX at UPG (---), MPG (—) and background (…) at MPG electrode.

2.3.3 Optimization of analytical parameters for NFX determination

The experimental parameters for the analysis of NFX at the MPG electrode were optimized in terms of MWCNT amount, pH, reaction time and square wave frequency, where the NFX concentration was kept constant (**Fig. 2.5)**.

The effect of MWCNT casting volume on the analytical performance of the MPG electrode was determined in the range between 5 – 50 µL (Fig. 2.5 A). The i_p increased with the increase in the volume of MWCNT casted from 5 – 30 µL. Over 30 µL, no increase in the current response was observed due to the saturation effect. Therefore, 30 µL volume of MWCNT was selected as the optimum volume for the fabrication of MPG electrode.

The adsorption time was optimized by dipping the MPG electrode into the same concentration of NFX (100 µM) for different lengths of time, ranging from 30.0 s to 9.0 min (Fig. 2.5 B). The current response increased with longer adsorption times from 30.0 s to 7.0 min but no significant increase in current was observed over 7.0 min possibly due to the saturation effect. Thus, optimized adsorption time for NFX determination was 7.0 min, which is quite rapid and well suited for its fast laboratory investigations.

The pH has a significant effect on the electro-reduction of NFX. The effect of pH for NFX detection was studied over a range of 2.1 – 8.0 (Fig. 2.5 C). A gradual shift in E_p to more negative

potential was observed with increase in the pH from 2.1 to 8.0. As the reduction of NFX involves consumption of hydrogen ions, the reduction becomes difficult with increase in pH and peak current decreases. The dependence of the E_p on pH of supporting electrolyte at MPG electrode is represented by the relation:

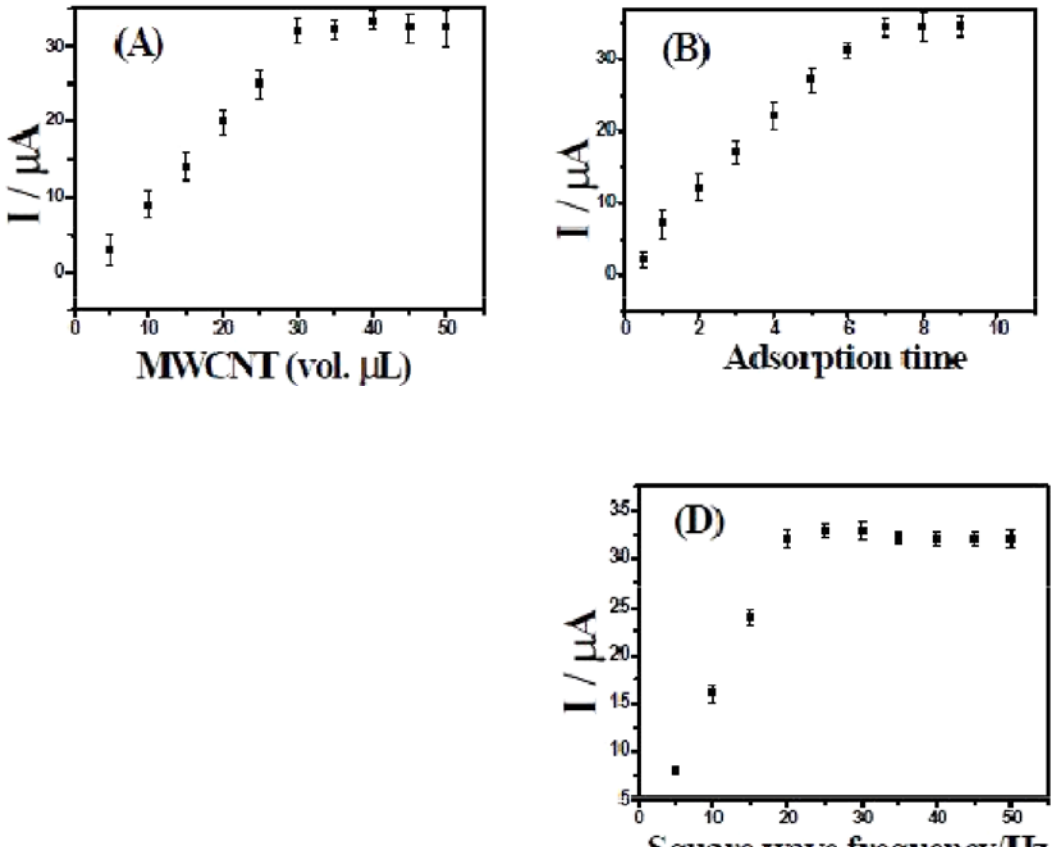
$-E_p$ (pH) = 58.89 pH + 1159.8 mV *vs* Ag/AgCl

with the correlation cofficient of 0.993. The slope value of ~ 59 mV pH^{-1} reveals that equal number of protons and electrons participate in the electrochemical reduction of NFX. Additionally, the current response was less at higher pH due to the insolubility of NFX at higher pH values. Thus, the maximum current was observed at pH 2.1 and it was used in the subsequent experiments and also to avoid over-potential exposure to the MPG electrode.

The dependence of cathodic peak current on square wave frequency was monitored in the frequency range 5 – 50 Hz (Fig. 2.5 D). The peak current increased linearly with increase in square wave frequency and the linear relation between i_p and f was expressed by the relation:

i_p (µA) = 1.028 f (Hz) + 10.95

at MPG electrode with the correlation coefficient of 0.973. The optimized parametrs of SWV were: initial potential: 0 mV, final potential: - 1600 mV, square wave frequency: 15 Hz, sensitivity: 100 µA/V, square wave amplitude: 20 mV and step potential: 4 mV.



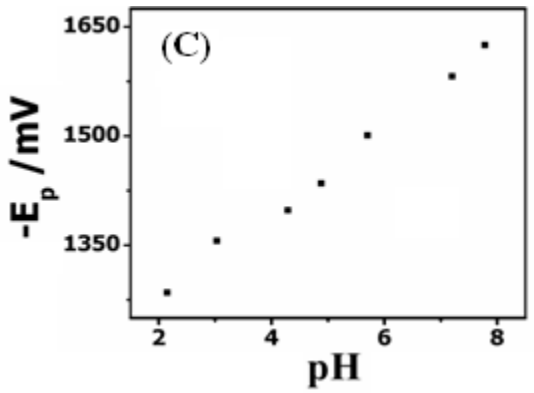


Figure 2.5: Optimization of experimental parameters; MWCNT volume (A), adsorption time (B), pH (C) and square wave frequency (D).

2.3.4 Analytical performance of the MPG electrode

2.3.4.1 Determination of NFX

Analytical performance of the MPG electrode was examined under the optimized conditions. The MPG was dipped into deoxygenated 0.1 M phosphate buffer not containing NFX (blank) and SWVs were recorded. No reduction peak was observed because NFX was not present in the test solution. Thereafter, the MPG electrode was reacted with different concentrations of NFX and SWVs were recorded. **Fig. 2.6** shows the SWVs recorded at MPG electrode at various concentrations of NFX.

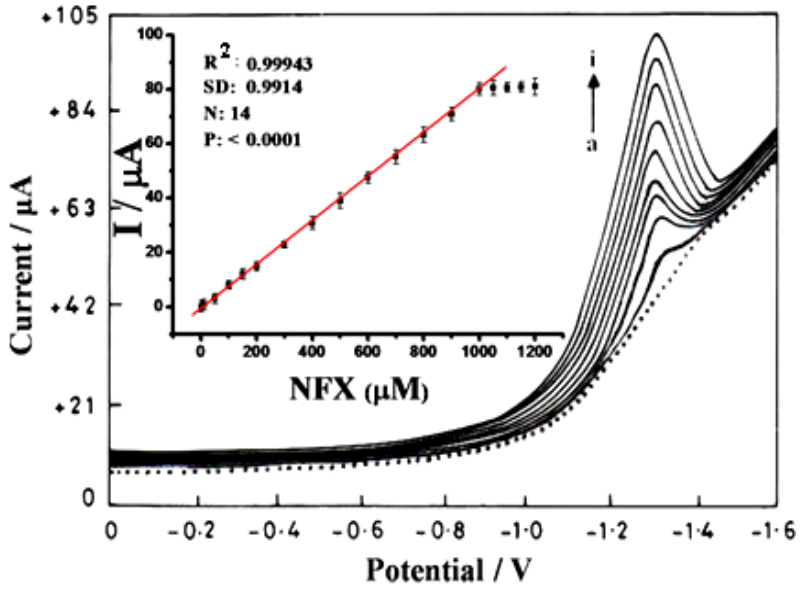


Figure 2.6: Square wave voltammograms of NFX at MPG electrode in phosphate buffer of pH 2.1. The curves were recorded at NFX concentrations a=1, b=100, c=200, d=300, e=400, f=500, g=600, h=700 and i=800 µM and background buffer of pH 2.15 is shown as (…). Inset shows the corresponding calibration plot.

A calibration plot (inset of Fig. 2.6) was obtained for NFX between 1.2 – 1200 µM with the dynamic range between 1.2 – 1000 µM. The linear regression equation is expressed as follows:

i_p (µA) = 0.072 [NFX µM] + 5.3281

with a correlation coefficient of 0.991. The detection limit for NFX was determined to be 40.6 ± 3.3 nM (R.S.D. < 5%) based on the measurements performed five times for the standard deviation of the blank solution (95 % confidence level, *k = 3*, *n = 5*). The sensitivity of NFX detection at MPG electrode was 0.072 µA μM^{-1}. The obtained detection limit is lower or comparable than the previously reported methods using electrochemical oxidation [24, 25] or chemiluminescence detection [26] of NFX in recent years. The detection limit is also comparable to a few electrochemical studies based on the reduction of NFX at mercury electrode [27, 28]. We have summarized the linear range and the detection limit of NFX by various methods in **Table 2.1**. The advantage of the present method for NFX determination is the operational negative potential window used, at which the common metabolites such as ascorbic acid, uric acid and dopamine etc, present in biological fluids do not interfere due to their non-reducible nature. However, a careful removal of dioxygen from the test solution is necessary to overcome its interference due to its reduction.

Table 2.1: A comparison of linear range and detection limit of norfloxacin by various methods.

Method	Linear range	Detection limit	Ref.
Spectrophotometric	6-62 µM	0.01 µM	1
Voltammetric (GCE)	15-50 µM	3.50 µM	2
Voltammetric (HMDE)	6-54 µM	0.02 µM	3
CuO/Nanotubes modified GCE	1-47.7 µM	0.32 µM	4
Solid phase spectro-fluorimetry	0.3-12 nM	0.1 nM	5
Fluorescence spectrometry	0.017-5.64 µM	5.0 nM	6

Nanotubes modified pyrolytic graphite	1.0-1000 µM	40 nM	This work

GCE - Glassy carbon electrode, HMDE - Hanging mercury dropping electrode

2.3.4.2 Determination of NFX in pharmaceutical tablets

The developed method was also tested for the determination of NFX in the commercial tablets. The tablets were dissolved in 0.5 mL of 0.1 N HCl and diluted with double distilled water so that the concentration of NFX lies in the working range. SWVs were then recorded under the optimized conditions and the NFX concentration in three tablets was found in good agreement with the reported values as shown in **Table 2.2**. The NFX recovery was found between 98.0 to 99.1 %, (R.S.D. ± 2.1 % for *n = 6*), indicating the applicability of this method in NFX determination in pharamaceutical samples.

Table 2.2: Comparison of observed and reported amount of norfloxacin in different medicinal tablets (n=3).

Samples	Amount reported (mg)	Amount observed ± S.D. (mg)	R.S.D. (%)	Recovery (%)
Norflox	400	399.01 ± 11.71	2 .93	99.75
Norflox-Tz	400	399.41 ± 12.32	3.08	99.85
Powerflox	400	400.13 ± 13.42	3.35	100.03

2.3.5 Analysis of NFX in clinical samples

The applicability of the MPG electrode for the determination of NFX was also determined by measuring its concentration in urine samples of five patients undergoing NFX treatment. The concentration of NFX was determined in the urine samples after 4 h of oral administration of single dose of Powerflox – 400. Prior to the analysis, the samples were diluted two times by buffer (dilution factor: 2X) and the pH was adjusted to 2.1. Initially a square wave voltammogram was

recorded for the urine sample of a healthy person (control). No peak was found in the normal urine sample as metabolites present in urine are non-reducible under the operational potential window. Urine samples of patients, undergoing treatment with NFX (4 h after oral administration of 400 mg NFX), were then used to determine the concentration of NFX. A well-defined reduction peak of NFX was observed at a peak potential of - 1285 mV in both the urine samples, indicating that the unmetabolized NFX is excreted in the urine samples under investigation. To further confirm that peak at - 1285 mV is due to reduction of NFX, the standard addition method was applied. Known concentrations of NFX were spiked, consequently the peak current increased linearly with increase in the sample concentration as shown in **Fig. 2.7 A**. Hence, it is concluded that the peak at - 1285 mV is due to the reduction of NFX, which is excreted in the urine samples of the patients. The concentration of NFX was determined using the calibration plot and was found to be 4.82 ± 0.31 µM. A similar value of NFX excreted has also been reported in literature based on the oxidation of NFX [29]. In another real sample experiment, midstream urine from a healthy individual was collected and filtered through a membrane filter. NFX spiked real sample solutions were prepared by adding NFX to the final concentrations of, 1.0, 10.0, 50.0, 100 and 500 µM in two times diluted urine samples. The recoveries of NFX from the spiked urine samples were calculated based on the calibration curve as shown in **Fig. 2.7 B**. The relative standard deviations were less than 3.7 % (*n = 5*). The results based on the recovery obtained clearly indicate that the NFX can be detected in the complex urine matrix without any interference.

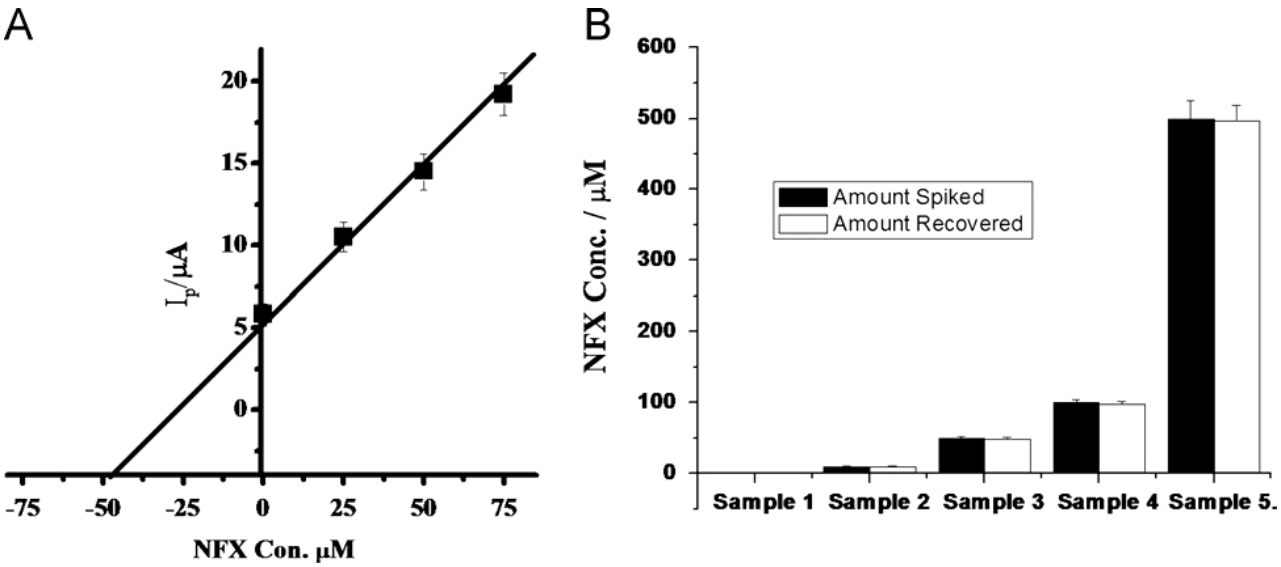


Figure 2.7: (A) Calibration plot for the determination of NFX concentration in the patients' urine samples (n = 5, R.S.D. < 4.1%) (negative value at x-axis is insignificant) and (B) Recoveries of NFX from the spiked urine samples (n=3, R.S.D. < 3.2 %).

2.3.6 Analysis of caffeine metabolism after NFX administration

In order to monitor the effect of NFX on caffeine concentrations, it is important to assign its detection potential. For this purpose, the SWVs for standard caffeine solutions were recorded at MPG electrode at various concentrations in phosphate buffers and urine samples (DF: 2X) of pH 7.2. A well defined oxidaion peak was observed at + 1.34 V and the i_p of the caffeine increased with increase in the concentration of caffeine. The peak current versus concentration plot was linear in the concentration range between 5 – 100 nM. The current values were obtained by subtracting the background current of the buffer solution and an average of atleast three replicate measurements was used to plot calibration curves. The linear regression equation for the calibration plot can be represented as:

i_p (μA) = 0.481 C (nM) + 0.261

having correlation coefficent of 0.989. To analyze the effect of NFX on caffeine metabolism, urine samples of five patients were tested. At first, square wave voltammogram of urine sample of a healthy person was recorded after dilution with buffer (DF: 2X) in the potential window of + 1.0 to + 1.8 V. No peak was found in the normal urine sample because the common metabolites present in urine are oxidizable below + 0.8 V [30]. The control samples were performed by analyzing the urine samples of persons taking only caffeine. A well defined oxidation peak of caffeine was observed at a peak potential of + 1.34 V and clearly indicated that unmetabolized caffeine is excreted in urine. To further confirm that peak at + 1.34 V was due to oxidation of caffeine, the standard addition method was applied. Known concentration of caffeine was spiked and it was observed that peak current increased linearly. Hence, it was concluded that peak at + 1.34 V is due to the oxidation of caffeine, which is excreted in the urine samples of the patients.

To examine the **e**ffect of prolonged NFX administration on the caffeine catabolism, urine samples were analyzed for the amount of caffeine after each day of NFX administration. The first urine sample in the morning was obtained from the patients after each day of NFX treatment. The patients were also taking two tablets of caffeine (200 mg/per day). The urine samples were diluted with buffer (1 : 1, DF; 2X) and SWVs were recorded. It was observed that the peak current

of caffeine was almost same after 1 day of NFX administration and the concentration of caffeine excreted was 2.5 ± 0.2 μM. However, with prolonged administration of NFX, the concentration of caffeine in urine increased with an increase in the NFX administration days. The peak current of caffeine in all urine sample became ~ 2.5 times higher (6.2 ± 0.2 μM) after 5 days of NFX administration as compared to 1 day administration as shown in the inset of Fig. 2.8. A comparison of caffeine concentration observed in different urine samples after 1 and 5 days of NFX administration is presented in **Fig. 2.8**. Caffeine concentration after 1 and 5 days NFX administration was reconfirmed by HPLC. The concentration of caffeine determined in urine samples by HPLC also showed similar trend as shown by electrochemical method using the MPG electrode. This clearly indicates that the NFX has prevented the caffeine catabolism (demethylation) in human body and facilates its accumulation, consequently high concentrations of caffeine appeared in the urine samples. Similar observation was reported earlier where caffeine concentration was increased after NFX administration during single blind clinical trial [3] and during liver microsomal incubations [31] in human urine and blood plasma. The inhibition of caffeine catabolism (or prevetntion of caffeine demethylation) by NFX leading to increase in caffeine in blood plasma of human system has also been reported by HPLC studies [4]. Thus, these studies confirm that the accumulation of caffeine in urine on prolonged tretament with NFX becomes ~ 2.5 times in comparison to 1 day of NFX administration. We have also performed the control experiment with volunteers without NXF administration but caffeine intake. In this case, the current value was less than what observed after 1 day of NFX administration (1.7 ± 0.01 mM). This was possibly due to the absence of NFX in the body which allows the catabolism of caffeine consequently lower concentrations of caffeine were detected in the urine samples. This observation clearly shows that NFX plays a pivotal role in the caffeine metabolism.

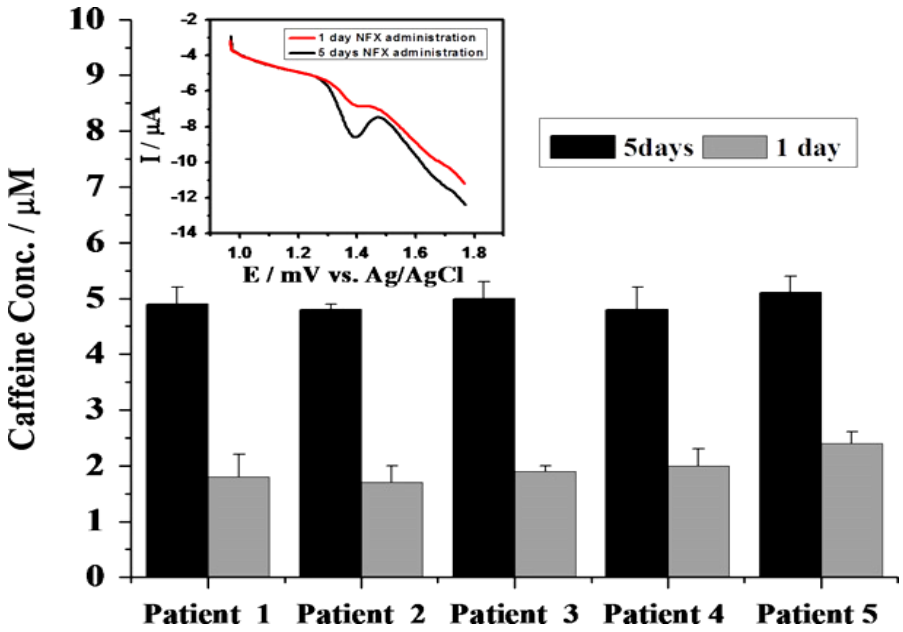


Figure 2.8: Histograms for the analyses of caffeine concentrations in the urine samples of patients (n=5) after 1 day and 5 days of NFX administration. Inset shows the comparative SWVs recorded in the patients urine samples 1 day after (red line) and 5 days after (black line) NFX administration.

2.3.7 Interference, stability and reproducibility study

The interference of ascorbic acid, uric acid and dopamine, the common metabolites present in urine and blood was examined by performing the analysis of 100 µM of caffeine in the presence of various concentrations of interferents (0.1 to 1.0 mM) under the optimized experimental conditions. As all these interferents under go oxidation below + 1.0 V, hence, they did not show any response in the potential range between + 1.0 to + 1.8V, consequently, a single oxidation peak of caffeine was observed. Similarly these metabolites do not interfere with determination of NFX as they do not undergo reduction. The interference studies carried out indicated that even 100 times higher concentration of interferents than caffeine and NFX did not cause any change in peak current of caffeine

The long term stability and reproducibility for the investigation of caffeine and NFX have also been investigated at MPG electrode. The electrochemical response of fixed concentration (100 µM) of caffeine and NFX was evaluated for a period of 10 days. Modified electrode was used daily and kept in the air. Experimental results show that a minimal decrease in current values was observed for caffeine with R.S.D. of 1.4 % and for NFX as 1.6 % for *n=5*, suggesting that the

modified electrode have excellent stability for the determination of caffeine and NFX. In order to check the intra-day reproducibility of the modified electrode six experiments were repeated for the same concentration (100 µM) of caffeine at pH 7.2 using the same MPG electrode. The results of six replicate measurements showed a RSD of < 1.4 % indicating the excellent reproducibility of results. These results demonstrated that MPG electrode is advantageous for the determination of caffeine as well as for NFX owing to its good stability and reproducibility.

2.4 CONCLUSIONS

We have selectively detected NFX in the patient's urine samples based on its electrochemical reduction using a solid MPG electrode for the first time. The modified electrode was successfully applied to monitor the caffeine catabolism (or prevention of caffeine demethylation) and its accumulation through the human urine samples analyses for the first time. The dynamic range for the NFX analysis ranged between 1.2 and 1000 µM with a detection limit of 40.67 ± 3.3 nM. The strategies described for the NFX determination and caffeine monitoring has many attractive features such as simplicity, rapidity and no requirement for specific labeling (i.e., a fluorescent or reactive moiety) and could be very useful in medical diagonistics. The ~ 2.5 times increase in the concentration of caffeine after prolonged NFX administration confirms the proof of concept of NFX pharmacology in terms of its ability to prevent the caffeine catabolism. The MPG electrode exhibited good stability and successfully detected NFX and caffeine in the clinical samples. The studies also further validate that the patients on medication with NFX should avoid overdose of caffeine so that accumulation of caffeine does not occur in body, which may lead to other medical complications.

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Chapter 3

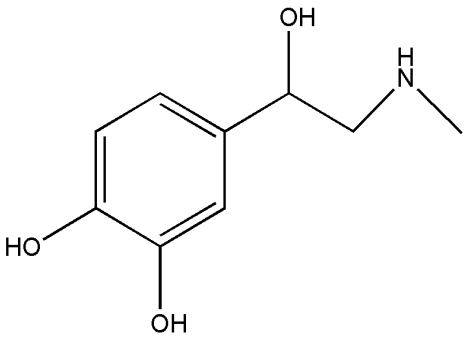
Heavy Ion Irradiation of MWCNT: A Study for the Electrochemical Determination of Neurotransmitters

3.1 INTRODUCTION

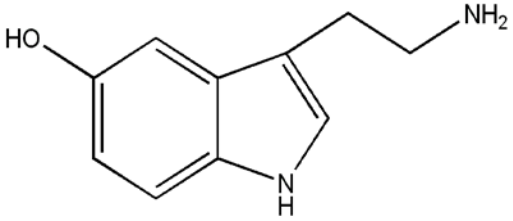
The swift heavy ion irradiation of carbon nanotubes is a very useful phenomenon for increasing the conductivity and surface area and can be very helpful for the electrochemical determination of biologically important molecules. It is observed that the mechanical properties, especially the stability of CNT has been found to be modified in controlled manner by ion beam treatment. The reason behind the intensive research on irradiation effects on these carbon nanomaterials are of the high technological importance and is attributed to their unique mechanical and chemical properties. By controlling the fluence of ions in ion beam irradiation technique, nano-carbons can be made suitable for variety of applications [1].

Neurotransmitters are the endogenous primary chemical messengers, which are released by the pre-synaptic nerve cells. They relay, amplify and transmit the signals to post-synaptic nerve cells and play a vital role in neuronal communication of central nervous system (CNS) [2, 3].

Epinephrine (1-(3,4-dihyroxyphenyl)-2-methylaminoethanol; EP) is an important catecholamine, which acts as a neurotransmitter in mammalian central nervous system for transporting the information between biological cells [4]. EP is secreted by medulla of adrenal gland in the situation of high psychological pressure or low blood sugar level and is used as drug to treat myocardial infarction, hypertension, bronchial asthma, cardiac reaction, severe allergic reaction and sepsis [5, 6]. EP, also called as adrenaline, is a hormone and plays an important role during the situation of mental stress. It stimulates series of actions of sympathetic nervous system called as "fight or flight response". EP concentration in blood affects the regulation of blood pressure and lipolysis, immune system, heart rate and glycogen metabolism [7, 8]. These important functions also make EP a potent doping agent; therefore, it is banned by the WADA during competitive games [9]. The monitoring of this catecholamine in human body fluids is of significant use for nerve physiology, medical diagnosis and especially for the patients suffering from Parkinson's disease, phaeochromocytoma and stress [10]. 5- Hydroxytryptamine (serotonin, 5-HT) is one of the major neurotransmitters of human brain and is known to play a central role in wide variety of pharmacological, biological and psychopathological functions including depression, eating disorders, alcoholism, obsessive-compulsive disorders and anxiety [11]. 5-HT is found in gastro intestinal tracts (GI) and CNS and acts both as neurotransmitter and a local hormone in peripheral vascular system in gut. A large amount of 5-HT in the body (over 90 %) is found in enterochromaffin (EC) cells of GI and stored in blood platelets, whereas, the brain contains only a minor proportion [12].



[Epinephrine]



[5-Hydroxytryptamine]

5-HT is synthesized from an essential amino acid tryptophan, a protein constituent of normal diet by tryptophan hydroxylase enzyme present in EC cells. 5-HT is implicated in various gastrointestinal disorders including irritable bowel syndrome, inflammatory bowel disease and food hypersensitivity [13]. In addition, neuro-degeneration of 5-HT has been found to associate with late-onset neurological diseases, including Parkinson's disease and Alzheimer's disease and possibly to normal aging of brain [14, 15]. Therefore, quantitative investigation of 5-HT in human urine and plasma is essential because of its coexistence in biological systems and regulating several physiological functions. Although, various techniques have been implemented for the determination of EP and 5-HT such as high performance liquid chromatography, spectro-photometric technique, capillary electrophoresis and flow injection analysis [16-18], however, these techniques require expensive instruments and time consuming pretreatment and derivatization processes which results in low recoveries and tedious procedure. Electrochemical techniques based on various approaches have been developed to overcome these difficulties [19-21]. The determination of EP has been reported at carbon paste and variety of other electrodes [22-24]. However, the simultaneous determination of EP and 5-HT by electrochemical methods remained a challenge due to the interference of other biomolecules like ascorbic acid, uric acid and dopamine present in biosystems. These interferents oxidize at a potential close to that of EP and 5-HT, resulting in an overlapping voltammetric response.

Therefore, the aim of the present investigation is to develop a sensitive voltammetric sensor for the simultaneous determination of EP and 5-HT in human fluids. A new approach based on irradiation of MWCNT by swift heavy Ag ions has been used to improve catalytic activity and conductivity. Carbon nanotubes are one of the most exciting materials these days due to their unique chemical and electronic properties and possess sp^2 carbon units which are in many nanometers in diameter and many microns in length [25]. The irradiation of carbon nanotubes by energetic Ag ions produce ion tracks (columnar defects) leading to the formation of amorphous carbon (*a-C*) [26, 27]. The sputtering of carbon atoms is also observed which produces the vacancies on the side walls and interistial atoms between the shells, providing the rough surface that leads to an increase in surface area of nanotubes thin film [28]. The size and hybridization of carbon system is also tailored by the ion beam treatment that makes ion beam irradiation a promising field of research [29-31]. In the present studies Ag ions of high energy (~ 120 MeV) at different fluence 1e12, 3e12 and 1e13 ions cm^{-2} were used for the irradiation of MWCNT. After

optimizing the experimental parameters, the irradiated sensor has been employed for the determination of EP and 5-HT in various human urine samples. The effect of common metabolites present in urine such as ascorbic acid, uric acid and dopamine has also been evaluated.

3.2 EXPERIMENTAL

3.2.1 Chemicals and reagents

EP and 5-HT were purchased from Sigma-Aldrich and used as received. Phosphate buffers were prepared by using the method of Christian and Purdy [32]. MWCNT (purity > 98 %) were received from Bucky, USA and used for the modification of ITO surface. ITO spurted glass sheets having size of 10 mm × 20 mm × 1.1 mm were obtained from Geomatec, Japan. Adrenaline bitartrate injections (G.K. Pharamaceuticals Ltd.) were obtained from the Institute Hospital of IIT Roorkee. All the reagents used were of analytical grades and double distilled water was used throughout the experiments.

3.2.2 Instrumentation

The voltammetric experiments were performed using a computerized BAS (West Lafayette, USA; CV-50W) electrochemical work station. A three-electrode cell system consisting of Ag/AgCl as reference electrode (3 M NaCl, Model MF-2052 RB-5B), platinum wire as counter electrode and irradiated MWCNT as working electrode, was used for the electrochemical measurements. The pH of the buffer solutions was measured using digital pH meter (Model CP-901). MWCNT modified ITO was irradiated using Pelletron Accelerator (15-UD) at Inter University Accelerator Centre, New Delhi, India. Raman spectra of un-irradiated (pristine) and 120 MeV Ag ions-irradiated MWCNT modified ITO were recorded using Renishaw in-via Raman microscope with Ar ion laser excitation at 514 nm at room temperature. FE-SEM instrument (JEOL-JSM 7400) was used to chracterize the surface morphology of pristine and irradiated electrode.

3.2.3 Irradiation of MWCNT

Suspension of MWCNT (0.5 mg mL^{-1}) was prepared in N,N-dimethylformamide solution and ultrasonic machine was used to acquire well dispersed suspension. A known volume (25 µL) of this solution was coated on the surface of bare ITO (10 mm x 10 mm) and was dried by evaporating the solvent at room temperature. MWCNT modified ITO was then irradiated using

120 MeV Ag ions at fluences of 1e12, 3e12 and 1e13 ions cm^{-2} with ion beam accelerator. The vacuum in the chamber during the irradiation was kept ~ 5 x 10^{-6} Torr. Electromagnetic scanner was used to scan the beam on the full area of thin film. The thickness of the carbon nanotubes layer was found to be bit larger as compared to the range of 120 MeV Ag ions in carbon. The sensor was prepared by putting the ITO between two scotch tapes and connected with copper strip for connections. A hole (3 mm diameter) on one side of the tape was made to expose the sensor for making the contact with solution. Time base technique was used to remove adsorbed analyte from the surface of irradiated MWCNT by applying a constant potential (-200 mV) for 1 min after each scan.

3.2.4 Experimental procedure

In order to prepare the stock solutions of EP and 5-HT (having concentration 1 mM), the desired amount of compounds was dissolved in double-distilled water. A known amount of them was added to 2.0 mL of supporting electrolyte. The total volume was made 4.0 mL with double distilled water. Square wave voltammograms were then recorded at the optimized parameters: initial potential: 0 mV, final potential: 1600 mV, square wave frequency: 15 Hz, square wave amplitude: 20 mV, step potential: 4 mV. Cyclic voltammograms were recorded in the potential range + 1.0 to - 1.0 mV at sweep rate of 10 – 500 mV s^{-1}. Urine and blood samples of healthy persons were obtained from Institute Hospital of IIT Roorkee and were stored in refrigerator immediately after collection. Blood sample was centrifuged at a speed of 1000 rpm for 5 min using EDTA as anticoagulant and supernatant blood plasma was used for the determination of EP and 5-HT. Urine and blood samples were suitably diluted to minimize matrix complexity. No further treatment was made. The standard addition method was employed for the determination of EP and 5-HT in real samples.

3.3 RESULTS AND DISCUSSION

3.3.1 Effect of irradiation on MWCNT

Raman spectroscopy is one of the widely used techniques for the structural characterizations of all forms of carbon; hence, it was used to determine the changes observed in MWCNT after irradiation. **Fig. 3.1** presents Raman spectra of 120 MeV Ag ion irradiated MWCNT and pristine at different fluences. It is observed that typical Raman spectrum of MWCNT shows first and second orders Raman scattering mode. The first order Raman scattering

mode; D-mode (disorder) was observed at ~ 1360 cm^{-1} and high energy G-mode (graphite) at ~ 1580 cm^{-1}. Second order overtone mode; D'-mode was observed at ~ 2700 cm^{-1} as reported in literature [26]. The position and intensity ratio of these modes are generally used to study the defect formation (damage) in MWCNT under energetic ion irradiation. The intensity of Raman modes was found to be largest for pristine sample and decreases with increasing fluence of 120 MeV Ag ions and can be explained on the basis of change in carbon cages of MWCNT after irradiation, leading to the formation of ion tracks.

Energetic ions during their passage lose energy by two independent means: elastic collisions between incoming ions and nuclei of the target atom (nuclear energy loss) and inelastic collisions between incoming ions and electrons of the target atoms (electronic energy loss). The energy lost by incoming ions is shared between the electrons of target atoms by electron-electron interaction and then transferred to the lattice atom by electron-phonon coupling. The irradiation results in localized heating of lattice along the ion path and high temperature cylindrical zone, the so called latent track is created. Temperature in this cylindrical zone is higher (thousands of Kelvin) than the melting temperature of material and this thermal spike quenches rapidly by dissipation of thermal energy from the ion tracks [33].

It is well known that in the case of carbon nanomaterials, the ion track consists of amorphous carbon material. The increase in the ion fluence (number of ions cm^{-2}) leads to passage of more number of Ag ions through the film and create more number of ion tracks with amorphous carbon material. Thus, with the increasing ion fluence, the electrical conductivity of the MWCNT film is expected to increase, probably due to the doping of Ag ions in the film, which should help in increasing the sensitivity of the detection method. However, the increase in ion fluence, also leads to damage in MWCNT and the catalytic activity decreases. Therefore, optimization of the fluence is necessary and in our case a fluence of 1e12 ions cm^{-2} has been used for the ordering of MWCNT at which minimum damage to MWCNT occurs.

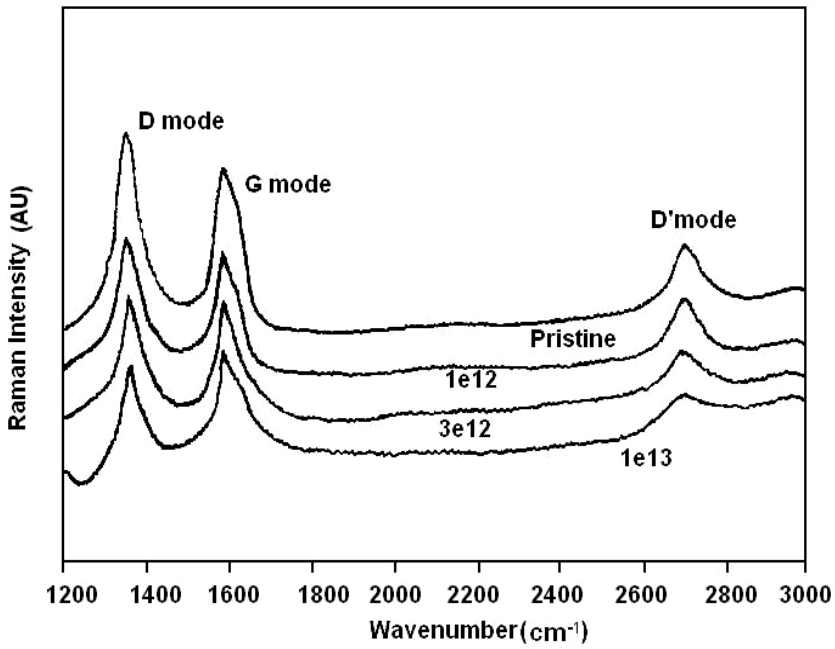


Figure 3.1: First and second order Raman spectra observed for pristine and 120 MeV Ag ion irradiated MWCNT at fluences of 1e12, 3e12 and 1e13 ions cm^{-2}.

Since low fluence irradiated MWCNT is expected to have better catalytic effect, the surface morphology of pristine and 120 MeV Ag ion irradiated MWCNT modified ITO with fluence of 1e12 ions cm^{-2} was studied using FE-SEM and the typical images are shown in **Fig. 3.2.** It is clearly revealed from these images that uniform dispersion of MWCNT was found in pristine, whereas, in energetic heavy ion irradiated MWCNT, nanotubes were found to be much scattered and a bit destroyed due to the impact of ion irradiation. The diameter of the MWCNT varies from 32 to 45 nm in the pristine and there is no significant change in the size of MWCNT after ion irradiation except some axial buckling was found. Due to ion irradiation, some MWCNT are damaged and their areal density is decreased. These observations support the results obtained from Raman spectroscopic measurements.

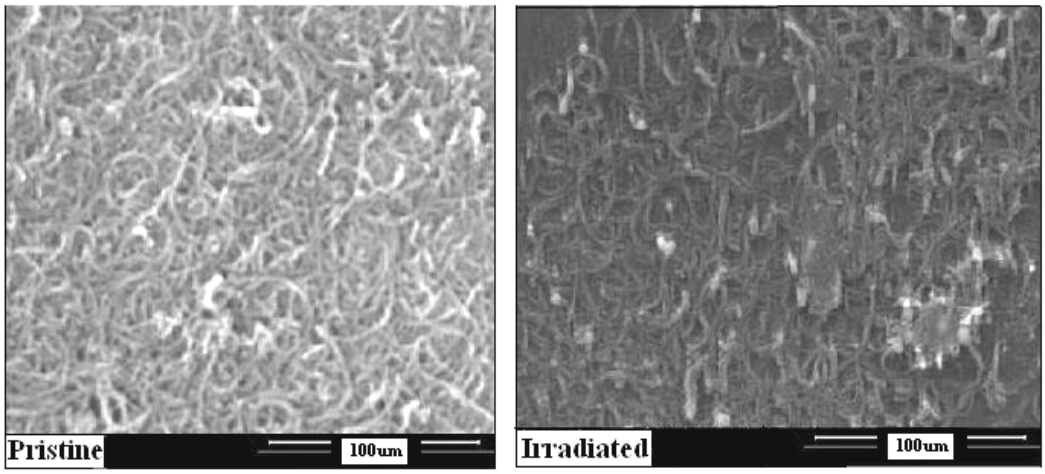


Figure 3.2: FE-SEM images observed for pristine and 120 MeV Ag ion irradiated MWCNT at fluence of 1e12 ions cm^{-2}.

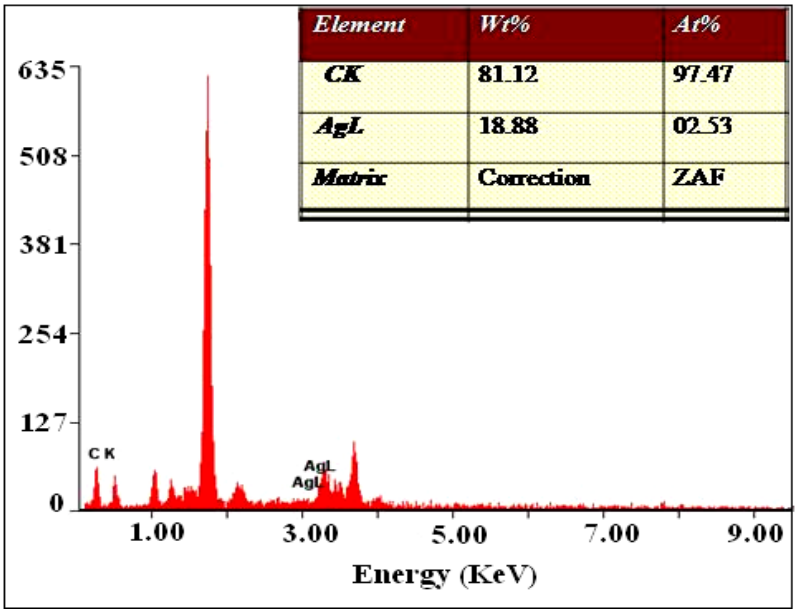


Figure 3.3: The EDAX spectrum of the MWCNT film, irradiated at a fluence of 1e12 ions/cm^2.

The typical energy dispersive X-ray analysis (EDAX) spectrum for the MWCNT film, irradiated at a fluence of 1e12 ions cm^{-2} indicated that peaks can be identified as arising from the film (Carbon and Silver). Analysis of the spectrum after background subtraction and convolution of peaks confirmed the composition of the irradiated film to be close to the 97.47 at. % C and 2.53

at. % Ag, as shown in **Fig. 3.3.** As film thickness is a bit larger as compared to the range of 120 MeV Ag ions in MWCNT film, the insertion of some Ag ions into the film is confirmed by EDAX spectrum.

3.3.2 Determination of effective surface area after irradiation

Difference in surface area of carbon nanotubes thin film after irradiation was calculated by recording cyclic voltammograms of 1 mM $K_3Fe(CN)_6$ at various scan rates using 0.1 M KCl as supporting electrolyte at pristine and 120 MeV Ag ion (with 1e12 fluence) irradiated MWCNT. A redox couple was found to notice due to the Fe^{+3}/Fe^{+2} at both the electrodes. Irradiated electrode exhibited a slight increase in i_p values and lesser value of peak-peak separation (ΔE_p) ~ 65 mV in comparison to pristine (~ 180 mV), showing the improvement in the reversibility of Fe^{+3}/Fe^{+2} redox couple, as shown in **Fig. 3.4 (A).**

The peak current for a reversible process follows the relation:

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

where i_p refers to the peak current (Ampere), F is Faraday's constant (96485 C mol^{-1}), R is the universal gas constant (8.314 J mol^{-1} K^{-1}), T is the absolute temperature (298 K), A is the surface area of electrode (cm^2), n = 1 for $K_3Fe(CN)_6$, D_R is diffusion coefficient (7.6×10^{-6} cm^2 s^{-1}), v is scan rate (Vs^{-1}) and C_0 is the concentration of $K_3Fe(CN)_6$ in mol L^{-1}. The surface area was calculated from the slopes of i_p versus $v^{1/2}$ plots and found as 0.085 and 0.184 cm^2 for the pristine and irradiated MWCNT modified sensor, respectively. Experimental results thus indicate that the surface area of irradiated MWCNT was ~ 2.15 fold larger than the surface area of the pristine, which is one of the important key factors for the detection of both neurotransmitters up to low detection limit.

3.3.3 Effect of fluence of ion beam on voltammetric response of neurotransmitters

High energy Ag ions with different fluences 1e12, 3e12 and 1e13 ions cm^{-2} were used to check the effect of fluence of heavy ion irradiation towards the voltammetric response of EP and 5-HT. At the same concentration of EP and 5-HT, voltammetric results observed were completely different at various kinds of fluences. In the case of 1e12 fluence, both compounds show sharp oxidation peak. While on increasing the fluence of irradiation, the peak current decreases and becomes minimum at fluence of 1e13. The high peak current at low fluence indicates the ordering

of carbon nanotubes, which are destroyed at high fluence as reported in literature [34]. Therefore, an irradiation of 1e12 fluence was choosen as an optimum fluence for further studies.

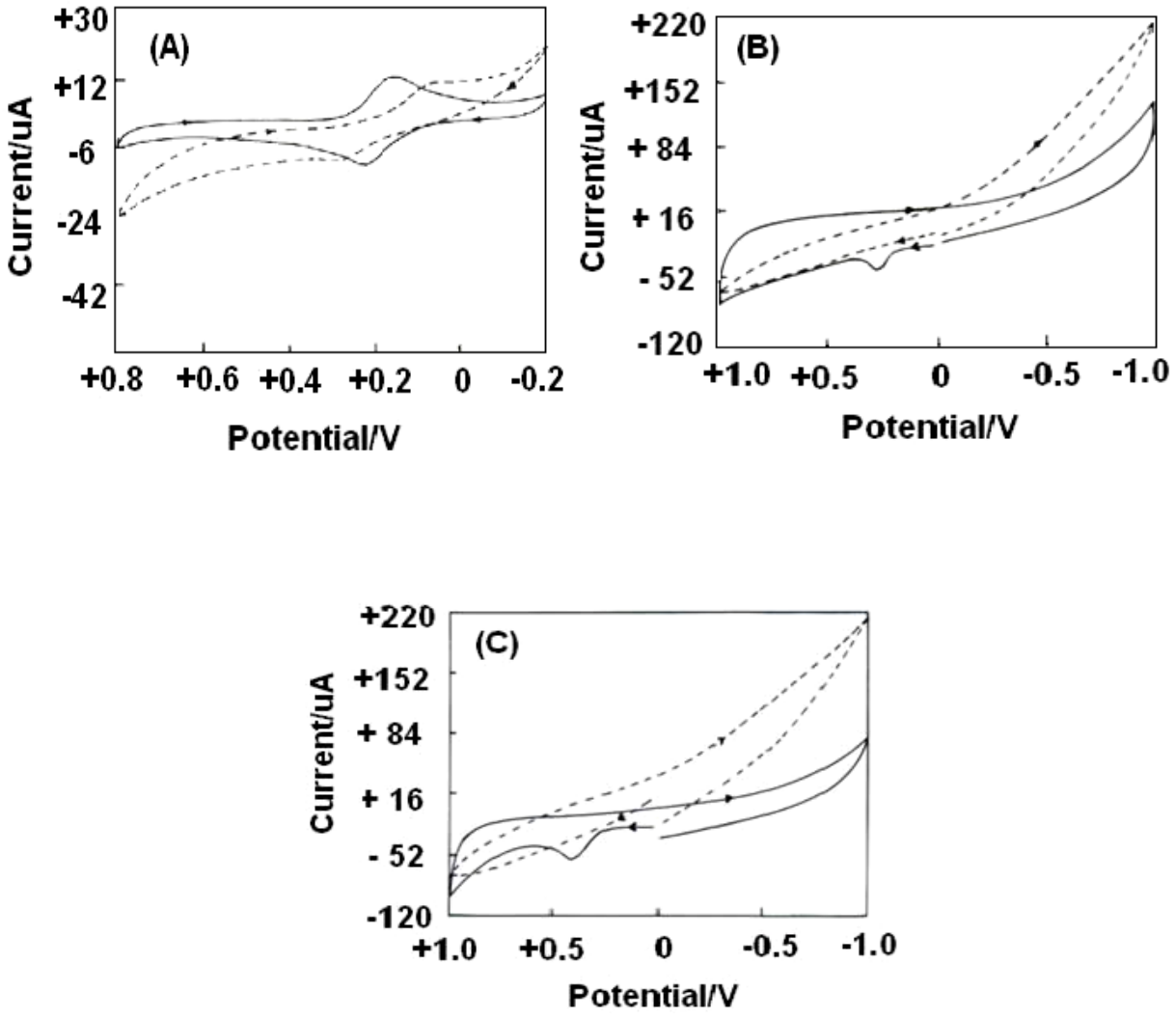


Figure 3.4: A comparison of cyclic voltammogram recorded at pristine (---) and Ag ion beam irradiated MWCNT/ITO (—) for (A) K_3 [Fe $(CN)_6$] in 0.1 M KCl, (B) EP, (C) 5-HT at pH 7.2.

3.3.4 Electrochemical investigation of epinephrine

3.3.4.1 Cyclic voltammetry

CV is the most important technique to establish the basic redox process of a compound. Hence, initially cyclic voltammograms of 25 µM EP at pristine and ion beam irradiated MWCNT

were recorded at pH 7.20 at a sweep rate of 50 mV s^{-1}. EP is irreversibly oxidized showing an anodic peak at ~ 250 mV at Ag ion irradiated MWCNT sensor, whereas at pristine no peak was observed **(Fig. 3.4 B)**. A significant increase in peak current suggests that irradiated MWCNT sensor acts as an efficient electron promoter to enhance the rate of electrochemical reaction of EP. To establish the nature of the electrode reaction, sweep rate studies were carried out in the range 10 – 500 mV s^{-1}. The peak current of EP was found to increase with increasing sweep rates and the linear plot of i_p versus $v^{1/2}$ clearly indicated that electron transfer process at irradiated sensor is diffusion controlled.

3.3.4.2 Square wave voltammetry

Square wave voltammograms of 100 µM EP were recorded at pristine and irradiated MWCNT. EP exhibited a sharp oxidation peak at pH 7.20 using irradiated sensor (E_p ~ 215 mV) and a small peak at pristine (E_p ~ 320 mV). The peak current of EP at irradiated sensor was nearly ten times larger than pristine. Thus, it is concluded that irradiated sensor catalyzes the oxidation of EP by increasing the peak current and shifting the peak potential to less positive potentials. The influence of pH of supporting electrolyte on the electrochemical response of EP at pristine and irradiated sensors was studied in the pH range 2.3 – 10.0. The peak potential of EP was dependent on pH and shifted to less positive potentials with increase in pH. The dependence of E_p on pH can be represented by the equations:

E_p (pH 2.15–10.0) = - 60.77 pH + 739.1 mV versus Ag/AgCl

E_p (pH 2.1 – 10.0) = - 60.26 pH + 634.5 mV versus Ag/AgCl

with R^2 of 0.990 and 0.992 at pristine and irradiated MWCNT, respectively. The value of dE_p/dpH ~ 59 mV pH^{-1} indicates that equal number of electrons and proton participate in the oxidation of epinephrine as reported in the literature [35].

Square wave frequency study of 100 µM EP was carried out in the frequency range 5 – 200 Hz at pristine and Ag ion irradiated MWCNT. The peak current corresponding to the oxidation of EP was found to increase linearly with the increase of square wave frequency. A linear relationship was found between peak current and square root of square wave frequency ($f^{1/2}$) at both the sensors and can be shown by the equations:

i_p (µA) = 3.461 $f^{1/2}$ + 0.60

i_p (µA) = 4.772 $f^{1/2}$ + 94.75

at pristine and irradiated MWCNT, respectively having correlation coefficients of 0.989 and 0.993. The linear relation between i_p and $f^{1/2}$ suggests that oxidation of EP at both the electrodes observes diffusion controlled path way [36, 37], which supports the results obtained using cyclic voltammetry.

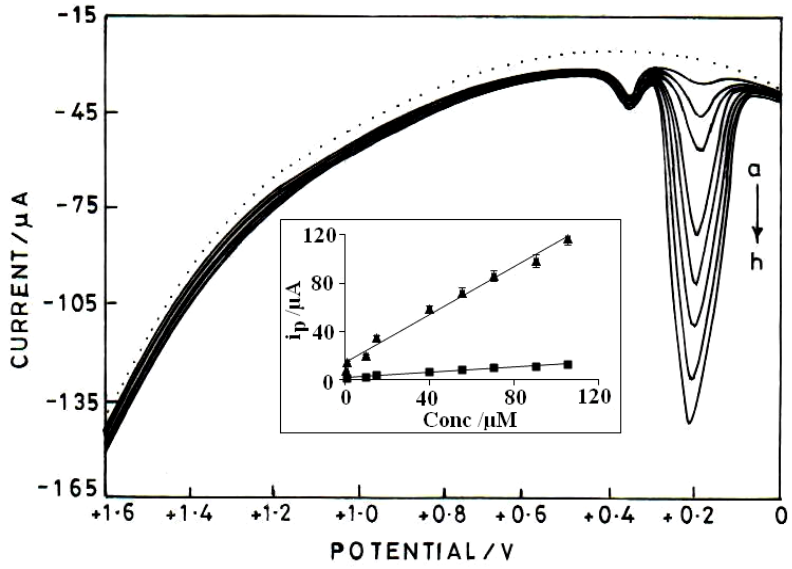


Figure 3.5: Square wave voltammograms observed for phosphate buffer (background) at irradiated electrode (…) and increasing concentration of EP at a fixed concentration (10 µM) of 5-HT; [EP]: a=0.1, b=10, c=15, d=40, e=55, (f) 70, (g) 90 and (h) 105 µM, The observed calibration curve for EP is shown in inset.

The determination of EP was carried out by varying the concentration of EP between 0.1 – 105 µM. The peak current of EP increased with increase in concentration of EP and the plot of i_p versus [C] was linear. The peak current values are obtained by subtracting the back ground current of buffer solution and the error bars are shown for three replicate measurements in the calibration plots, as depicted in inset of **Fig. 3.5**. The linear relation between peak current and concentration at pristine and irradiated MWCNT can be expressed by the relations:

i_p (µA) = 0.108 [C] + 2.01

i_p (µA) = 0.998 [C] + 13.91

having correlation coefficients of 0.985 and 0.986, respectively, where [C] is the concentration of EP in µM. The detection limits and sensitivity of EP for both pristine and Ag ion beam irradiated sensors were found to be 100 nM, 2 nM and 0.108 µA μM^{-1}, 0.998 µA μM^{-1}, respectively.

3.3.5 Electrochemical investigation of 5-HT

3.3.5.1 Cyclic voltammetry

Cyclic voltammograms of 25 µM 5-HT at scan rate of 50 mV s^{-1} were recorded in phosphate buffer of pH 7.20 at pristine and irradiated sensors. No electrochemical signal was observed corresponding to the oxidation of 5-HT at pristine, whereas, a well defined oxidation peak (E_p ~ 392 mV) was observed at irradiated MWCNT (**Fig. 3.4 C**). These results clearly indicate that catalytic activity of MWCNT is enhanced upon irradiation by Ag ions. The absence of anodic peak in reverse scan clearly reveals that oxidation of 5-HT is irreversible. To ascertain the nature of the electrode reaction, sweep rate studies were carried out in the range 10 – 500 mV s^{-1} at irradiated sensor. The peak current was found to increase with increasing sweep rates and the linear plot of i_p versus $v^{1/2}$ clearly indicated that oxidation of 5-HT is diffusion controlled.

3.3.5.2 Square wave voltammetry

The square wave voltammograms of 100 µM 5-HT at pristine and irradiated sensors were recorded in phosphate buffer media of pH 7.20. In the case of pristine a small peak is observed (E_p ~ 450 mV), which shifts to less positive potential (E_p ~ 360 mV) at irradiated sensor as shown in **Fig. 3.6**. A remarkable enhancement in peak current is observed at irradiated sensor in comparison to pristine, which can be attributed to the increased conductivity of MWCNT upon irradiation. The effect of pH on the anodic peak potential of 5-HT was studied in the pH range 2 – 10. It was found that E_p shifts to less positive potentials with increase in pH value. The plots of E_p versus pH were linear at pristine and irradiated MWCNT and obey the relations:

E_p (pH 2.15 – 9.96) = - 36.36 pH + 700.4 mV versus Ag/AgCl

E_p (pH 2.15 – 9.96) = - 37.46 pH + 619.5 mV versus Ag/AgCl

having R^2 of 0.993 and 0.990, respectively. The slope of 36 and 37 mV per pH unit indicates that number of protons and electrons involved in oxidation mechanism of 5-HT is unequal and only one proton is involved in two electron oxidation of 5-HT [11, 38].

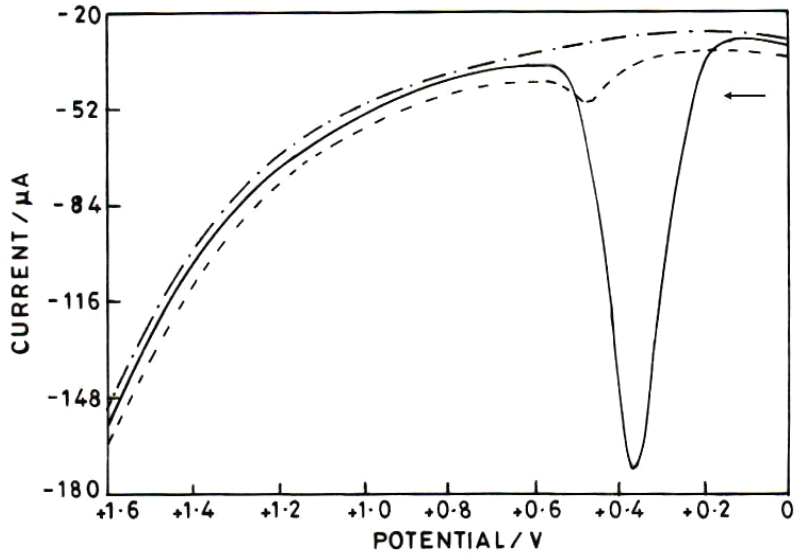


Figure 3.6: Comparison of square wave voltammogram for 100 µM 5-HT at pristine (---), ion irradiated MWCNT (—) and background phosphate buffer (—•—) at pH 7.20.

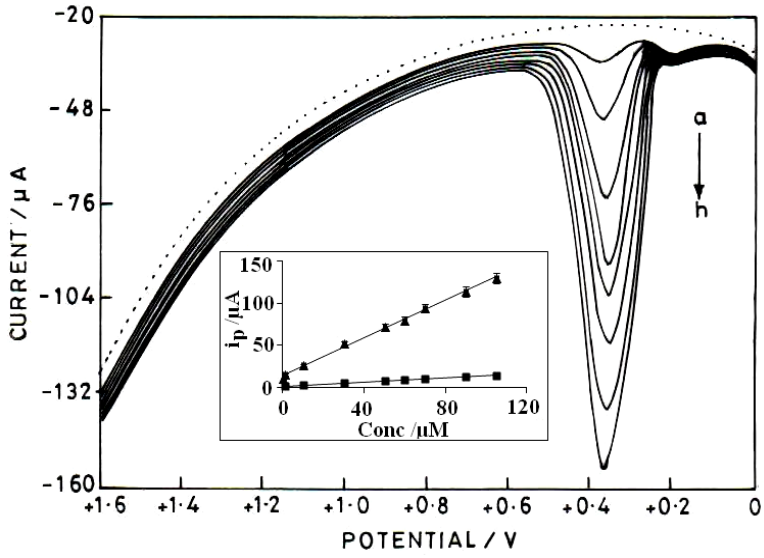


Figure 3.7: Square wave voltammograms observed at a fixed concentration of EP (10 µM) with increasing concentration of 5-HT; (a) 0.1, (b) 10, (c) 30, (d) 50, (e) 60, (f) 70, (g) 90 and (h) 105 µM. The background at irradiated electrode is shown as (---) and inset shows calibration curve for 5-HT.

The effect of square wave frequency on 100 µM 5-HT was examined by varying the frequency in the range 5 – 200 Hz at pristine and 120 Ag ion irradiated sensors. The lineaer relation observed

for peak current versus $f^{1/2}$ at pristine and irradiated MWCNT can be presented by the equations, respectively:

i_p (μA) = 3.316 $f^{1/2}$ + 2.602

i_p (μA) = 4.702 $f^{1/2}$ + 101.46

and confirms the electrode reaction of 5-HT as diffusion controlled [11].

For quantitative analysis of 5-HT, square wave voltammograms at different concentration of 5-HT were recorded at pristine and irradiated MWCNT sensors in the range 0.1 – 105 μM. The peak current was found to increase systematically with increase in the concentration of 5-HT as shown in **Fig. 3.7**. Linear regression equations arising from calibration plots (inset of Fig. 3.7) for pristine and irradiated MWCNT can be expressed as:

i_p (μA) = 0.120 [C] + 1.722

i_p (μA) = 1.119 [C] + 14.18

having correlation coefficients of 0.997 and 0.999, respectively, where [C] is the concentration of 5-HT in μM. The detection limit of 5-HT was found to be 80 nM and 0.75 nM for pristine and ion beam irradiated MWCNT, respectively.

3.3.6 Simultaneous determination of EP and 5-HT

The simultaneous determination of EP and 5-HT was carried out at ion beam irradiated MWCNT. Square wave voltammograms were recorded for different concentration of EP and 5-HT when both the compounds are present in the same solution. In the first set of experiments, 5-HT was kept at fixed concentration (10 μM) and the concentration of EP was varied in the range 0.1 μM to 105 μM. In the second set, the concentration of 5-HT was varied in the range 0.1 μM to 105 μM keeping the EP concentration constant at 10 μM. It was observed that the peak current of EP increased with an increase in EP concentration when 5-HT concentration was kept constant. The peak current of 5-HT remained practically constant. Similarly on keeping the concentration of EP constant, the peak current of 5-HT increased with increase in concentration of 5-HT, while that of EP did not change. The dependence of i_p on concentration in each case obeyed the same relation as was observed in the individual determinations. From the experimental results described above, it can be seen that EP and 5-HT can be easily determined in the presence of each other. The current responses of EP and 5-HT further proved the utility of this irradiated sensor for the simultaneous

determination of two species by changing the concentration of EP and 5-HT in the range 10 – 100 μM in the mixture at the same time.

3.3.7 Analytical applicability

3.3.7.1 Pharmaceutical analysis

Ion beam irradiated MWCNT was applied for the determination of EP in adrenaline bitartrate injection (specified content of EP; 1 mg mL^{-1}). An adequate content (0.1 mL) of this sample was diluted to 10 mL with phosphate buffer of pH 7.20 so that it falls into linear concentration range. Square wave voltammograms were then recorded under optimized parameters. EP content present in the injection exhibited oxidation peak (E_p ~ 215 mV) at the same potential as exhibited by the standard EP solution. The EP injection sample was then spiked with different concentration of standard EP solution. The results obtained from recovery experiments in the range 98 – 105 % are summarized in **Table 3.1**. The average determination results of EP in the injection were 1.02 mg mL^{-1}, which resemble to the value given on injection specifications. This process was repeated eight times and the relative standard deviation obtained was 2.0 %. Therefore, it is concluded that proposed sensor can be utilized successfully for the determination of EP in pharmaceutical preparations.

3.3.7.2 Urine sample analysis

The simultaneous determination of EP and 5-HT was then carried out in urine samples of healthy persons using Ag ions irradiated MWCNT. Two urine samples were collected from healthy personnel and diluted 4 times with phosphate buffer of pH 7.20. Square wave voltammograms of urine sample at irradiated electrode were recorded and is shown in **Fig. 3.8**. Two distinguished peaks were observed at ~ 215 and ~ 360 mV corresponding to the oxidation of EP and 5-HT and an additional peak was noticed at ~ 285 mV and was identified due to uric acid (UA). The urine samples were then spiked with the standard concentration (0.01, 0.02 and 0.03 μM) of EP and 5-HT for confirmation. The results obtained from the determination of EP and 5-HT in the urine samples are summarized in **Table 3.2** and are essentially similar to the values reported in literature [39, 40]. As the detection limits for EP and 5-HT using irradiated sensor is in nM level, it became possible to detect them in healthy human urine samples. The concentration of catecholamines and 5-HT generally increase in patients suffering from carcinoid syndrome and essential hypertension

and hence, it can be easily determined at irradiated MWCNT without any interference commonly present in blood and urine.

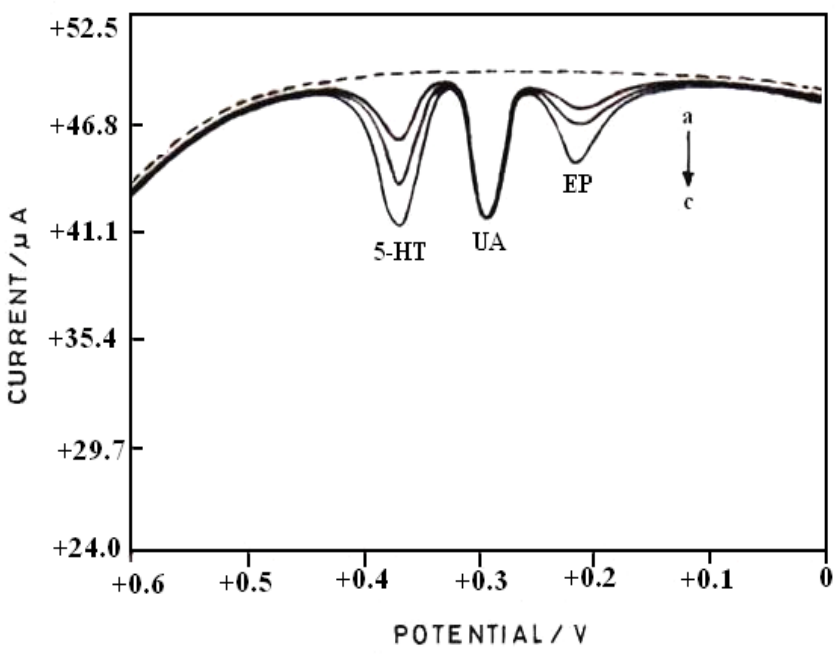


Figure 3.8: Square wave voltammograms observed for simultaneous determination of EP and 5-HT in healthy human urine sample; (a) urine sample showing peaks of EP and 5-HT; (b and c) after spiking with standard EP and 5-HT; The dashed line shows blank at pH 7.20.

Table 3.1: Observed results for EP determination in EP injections at Ag ion irradiated MWCNT.

Sample	Content (mol L^{-1})	EP added (mol L^{-1})	EP found (mol L^{-1})	Recovery %
1	4.50×10^{-5}	–	4.55×10^{-5}	101.11
2	4.50×10^{-5}	1.5×10^{-5}	6.25×10^{-5}	104.16
3	4.50×10^{-5}	2.0×10^{-5}	6.73×10^{-5}	103.54
4	4.50×10^{-5}	2.5×10^{-5}	6.92×10^{-5}	98.86

3.3.7.3 Blood sample analysis

Altered concentration of EP and 5-HT in serum affects the function of central nervous system so it is very necessary and useful to monitor their level in blood serum. Several possible interfering substances such as glucose, vitamins, dopamine and oxalate were examined for their impact on the determination of EP and 5-HT. It was found that all the species with a concentration of 10 μM do not interfere for the measurement of 0.1 μM EP and 5-HT. Ag ion irradiated MWCNT/ITO was applied for detection of EP and 5-HT in blood sample of healthy person. Standard addition technique was used for this purpose. The 5-HT concentration in serum was detected as 0.15×10^{-6} μM, which is fairly close to the concentration reported in literature [41]. No EP was detected in the blood sample of healthy person. With exogenous spiking of EP and 5-HT (0.05, 0.1, 0.2 and 0.4 μM), recovery was found in the range 97 – 103 % for EP and 101 – 104 % for 5-HT. The results clearly demonstrate the capability of ion beam irradiated MWCNT sensor in the voltammetric determination of EP and 5-HT in blood serum samples with high selectivity, accuracy and good reproducibility.

3.3.8 Stability and reproducibility of irradiated electrode

Stability of 120 MeV Ag ion irradiated MWCNT/ITO electrode towards EP and 5-HT determination was evaluated by measuring current response at fixed concentration of 10 μM EP and 5-HT in phosphate buffer of pH 7.20. Electrode was used daily upto 10 consecutive days and stored in air. The results showed that irradiated electrode exhibited current response with RSD of 4.5 % and 3.95 % for EP and 5-HT determinations, respectively. This suggests that this electrode possess good stability for the determination of EP and 5-HT.

The intra-day reproducibility of ion beam irradiated electrode was also measured. Five repetitive measurements were carried out for 10 μM EP and 5-HT solutions on the same day to show intraday reproducibility. Voltammetric responses were obtained with RSD of 0.76 % and 0.99 % for EP and 5-HT, respectively. Thus, it is concluded that irradiated MWCNT sensor has good reproducibility.

Table 3.2: Observed results for EP and 5-HT determination in human urine sample at Ag ion irradiated MWCNT.

	Sr. no.	Amount added (µM)	Amount detected (µM)	Recovery (%)
EP	1	0.000	0.062	–
	2	0.010	0.070	97.22
	3	0.020	0.093	101.08
5-HT	1	0.000	0.250	–
	2	0.020	0.271	100.37
	3	0.030	0.302	100.66

3.4 CONCLUSIONS

A novel electrochemical sensor has been developed for the first time using swift heavy Ag ion irradiation phenomenon to enhance the sensitivity of MWCNT. The irradiation not only increased the effective surface area of the sensor by ~ 2.5 times but also the insertion of Ag ions in the MWCNT film caused the electrocatalytic effects. The 120 MeV Ag ions irradiated MWCNT sensor exhibited strong catalytic effects towards the oxidation of EP and 5-HT. Moreover, irradiated sensor resolved the overlapping anodic peaks of EP and 5-HT into two well-defined peaks. A comparison of detection limits reported in literature in last few years for EP and 5-HT **(Table 3.3)** clearly indicates that the dynamic range and detection limit observed in the present studies is comparable or better than the reported ones [21-23, 42-44]. The method provided detection limit values much lower than the concentration of these compounds present in human urine. Thus, detection of these neurotransmitters released in urine can serve as a tool for the diagnosis of several diseases. The irradiation of carbon nanotubes by swift heavy ions is likely to associate with the production of localized defects including amorphization in nanomaterials. Irradiation causes an increment in the conductivity of nanotubes thin film due to the insertion of Ag ions. It is found that disorder parameter (I_D/I_G) first decreases at low fluence and then increases at high fluence, which confirms the ordering of MWCNT by ion irradiation [45]. Among 1e12,

3e12 and 1e13 fluence, the best results were obtained at 1e12 and hence, this fluence was chosen as an optimum fluence for exhibiting the catalytic effect. Results obtained from the application of proposed protocol for determining EP and 5-HT in real samples such as urine, blood and pharmaceutical samples confirmed the good accuracy and precision of proposed method. The coexisting ascorbic acid, dopamine and uric acid do not exhibit any interference for the simultaneous determination of EP and 5-HT. Excellent features, like wide linear range, low detection limits, high reproducibility and repeatability and long term stability proved the successful application of this irradiated MWCNT sensor for the determination of neurotransmitters in pharmaceuticals and biological fluids.

Table 3.3: Comparison of dynamic range and detection limit of the Ag ion irradiated sensor with other sensors reported in the literature.

Electrode	Compound	Linear range	Detection limit	Ref.
Gold nano clusters/Polypyrrole/GCE	EP	0.3 – 20 µM	30 nM	[21]
PGE/CNT	EP	0.5 – 100 nM	1.5 nM	[22]
Carbon paste/CNT Carbothiamide	EP	50 nM–550 µM	9.4 nM	[23]
Carbon paste/Iron complex	5-HT	1 – 15 µM	1 µM	[42]
Carbon paste/Iron Complex	5-HT	0.5 – 8.8 µM	0.2 µM	[43]
Gold nano cluster/ Polypyrrole	5-HT	7 nM – 2.2 µM	1.0 nM	[44]
Ag ion Irradiated MWCNT/ITO	EP	0.1 – 105 µM	2 nM	This work
	5-HT	0.1 – 105 µM	0.75 nM	

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Chapter 4

SWCNT Modified Sensor for the Investigation of Halobetasol; a Topical Corticosteroid

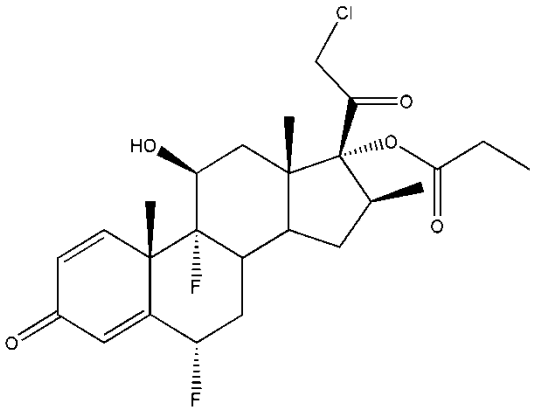
4.1 INTRODUCTION

Halobetasol propionate (6α, 11B, 16B)-21-chloro-6,9-difluoro-11-hydroxy-16-methyl-17-(1-oxopropoxy)pregna-1,4-diene-3,20-dione (I, HBP) is a topical corticosteroid, which is widely used as anti-inflammatory, antipruritic and vasoconstrictive agent [1, 2]. Topical corticosteroids are absorbed through the skin cells. They prevent these cells to generate different inflammation-causing chemicals, which are released on the reaction of skin to irritation and allergens [3, 4]. HBP is an ultra high-potency corticosteroid and is, therefore, suitable for the treatment of patients suffering with severe, localized corticosteroid-susceptible dermatoses such as plaque psoriasis, lichen simplex chronicus and severe atopic dermatitis [5, 6]. Psoriasis is a chronic inflammatory skin disorder of complex origin and two to three million people in the United State and approximately 1 % of the world population is affected by this disease [7]. Since psoriatic plaques are thick, scaly, indurated and dry, HBP is found to be very effective in the treatment of chronic and localized plaque psoriasis [8].

However, the use of HBP is associated with several side effects such as atrophy, postulation, leukoderma, acne, millaria, parasthesia, telangiectase, urticara and striae [9]. Therefore, a number of studies have been reported, explaining the analytical methods for determination of HBP. Reported methods for the investigation of this drug include spectrophotometric methods [1], polarographic method [10], high performance liquid chromatography and high performance thin layer chromatography [11]. Most of the methods reported suffer from many disadvantages such as long separation time, necessity of expensive instruments, complicated procedure and several derivatization steps are required prior to approach final analysis. Therefore, it is required to develop a simple, selective, sensitive and inexpensive technique for the determination of compound I.

In recent years voltammetric techniques have demonstrated the advantages of being both rapid and economical in the determination of several organic and inorganic compounds with high sensitivity and low detection capability. In addition, electrochemical methods offer ease of operation and simple instrumentation. These methods are found less sensitive to matrix complexity than other conventional analytical techniques [12, 13]. It has been found that the utilization of modified electrode augments the sensitivity for electrochemical determination of biomolecules and drugs [14-16]. Searching the published methods for the determination of HBP shows that no electrochemical study has been performed till now. One of the reasons for this is the fact that the

reduction of HBP occurs at high negative potential, which consequences in merging of the signal current with the background current. In all electrochemical measurements, the reaction of interest occurs at the surface of working electrode; therefore, the selection of working electrode can be a powerful tool for the success of any electrochemical reaction.



[Halobetasol Propionate]

Recently we have advocated the use of EPPGE for broad use in electroanalysis as it provides low background current, wide potential range in both positive and negative directions and improved elecrocatalytical signals in comparison to other conventional electrode [17, 18]. SWCNT have been utilized to improve electrocatalytic resoponse of electrode and such inherent properties of SWCNT are believed to be favorable towards the redox reaction of electrochemical species by shifting the peak potential in less positive or negative direction with enhancement of peak current simultaneously. Carbon nanotubes with their extraordinary electrochemical and mechanical properties have drawn much attention during last decade. Owing to huge surface area, subtle electronic characteristics and strong adsorptive capability, CNT have the capability to promote the electron transfer reactions of electroactive bimolecules and drugs [19-21]. A comparison of catalytic activity of SWCNT and MWCNT for the determination of amlodipine besylate and other biomolecules has been made [22, 23] and it is found that SWCNT exhibits higher activity as compared to MWCNT; hence, this article reports a convenient method for the assay of HBP based on unique characteristic of SWCNT. At SWCNT coated EPPGE the electrochemical response of HBP improves remarkably as reduction peak current increases and peak potential shifts to less

negative potential as compared to bare EPPGE. After optimizing the experimental parameters, developed procedure has been used for the direct determination of HBP in pharmaceutical formulations. The product of reduction has been characterized by IR and NMR and the possible site of >C=O has been deduced.

4.2 EXPERIMENTAL

4.2.1 Instrumentation

All the voltammetric measurements were performed using BAS (West Lafayette, USA; CV-50 W) voltammetric analyzer. The electrochemical cell used was a single compartment glass cell equipped with Ag/AgCl (3M NaCl, model BAS MF-2052 RB-5B) as reference electrode, a platinum wire as counter electrode and SWCNT/EPPGE as working electrode. The pyrolytic graphite plates and pieces were obtained from Pfizer Inc., New York, USA. Phosphate buffers in the pH range (2.3 – 9.9) were prepared and the pH was measured using digital pH meter (model CP-901). Surface morphology of the bare and SWCNT/EPPGE was studied by using FE-SEM (JEOL JSM-7400) instrument.

CPE was carried out in a three-compartment cell consisting three electrode system using pyrolytic graphite plate (area 6 × 1 cm^2) as working electrode, platinum gauge (cylindrical) as an auxiliary electrode and Ag /AgCl as reference electrode. UV-vis spectral studies were carried out using spectrophotometer (Perkin-Elmer Lambda 35). The FT-IR spectra were recorded with the help of a spectrophotometer (Perkin-Elmer 1600 series) using KBr pallets. GC-MS analysis was carried out with spectrometer (Perkin Elmer Clares 500 EI mode) using HP-17 column at 70 eV. ^{1}H-NMR spectral studies were performed in an appropriate deuteriated solvent ($CDCl_3$) with $SiMe_4$ (internal standard) using Advance 500 Digital NMR, Brucker. Chemical shift (δ) values have been indicated in parts per million (ppm).

4.2.2 Reagents and materials

HBP in powdered form was acquired as a gift from Symbiotic Pharma labs Ltd. Ankleshwar, India. SWCNT of purity > 98% were received from Bucky, Houston, TX, USA. The SWCNT was analyzed for metal contents using atomic absorption spectroscopy and the Fe, Co and Ni were found as 0.819, 0.412 and 0.207 %, respectively. Phosphate buffers of various pH and ionic strength were prepared by using the method reported by Christian and Purdy by mixing the standard solutions of Na_2HPO4 and NaH_2PO4 [24]. HBP containing creams and ointments of

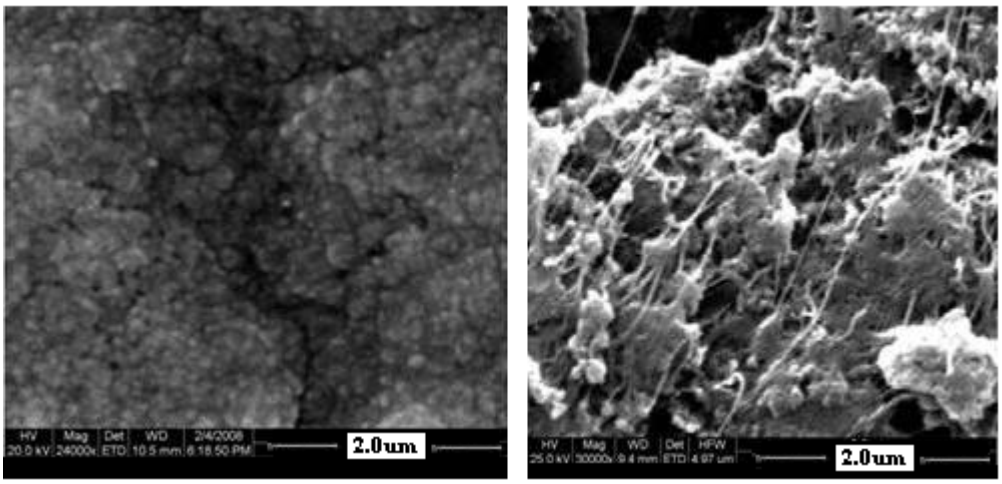
different companies were obtained from the local market of Roorkee. The medicinal samples (cream) of HBP obtained were Halovate (Glenmark pharmaceuticals Ltd., Mfg. Lic. No. MNB/05/182), Halox (Ranbaxy Labs. Ltd., Mfg. Lic. No. G/632) and Halobet-S (Ajanta Pharma Ltd., Mfg. Lic. No. KD-2590-A). All the chemicals, which were used for analytical measurements were of analytical grade and were obtained from Merck. The double distilled water was used for all the experiments carried out.

4.2.3 Preparation of bare and SWCNT modified EPPGE

A pyrex glass tube of suitable 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. The piece (2×3 mm^2) was then inserted in glass tube carefully from the other open end of the 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.

Modification of EPPGE was carried out by rubbing it on emery paper (P-600) followed by cleaning it with double distilled water. The *N, N*-dimethyl formamide was used for suspension of SWCNT and suspension was prepared by dispersing 0.5 mg of SWCNT in 1 mL of DMF by ultrasonic agitation. Amount of nanotubes casted on the electrode surface has a great significance towards the electrode response. Hence, different amount of nanotubes were casted in the range 5 – 40 µL at the electrode suface and electrode response was checked by recording voltammograms. Peak current was found to increase with increase in the volume of nanotubes casted up to 25 µL and then remained constant up to 40 µL. Therefore, 25 µL was selected as an optimum amount of SWCNT for modification of electrode. This optimum amount of SWCNT suspension was coated on to the surface of bare EPPGE and was allowed to evaporate at room temperature. This modified electrode was used for further experiments. The surface morphology of bare and SWCNT/EPPGE

was characterized by recording FE-SEM using Quanta 200 FE-SEM instrument. A comparison of FE-SEM images of the bare and SWCNT-modified EPPGE has been shown in **Fig. 4.1**.



(A) **(B)**

Figure 4.1: Typical FE-SEM images of (A) bare EPPGE and (B) SWCNT/EPPGE.

4.2.4 Voltammetric Procedure

Halobetasol Propionate is insoluble in water; hence, stock solution of HBP was prepared by dissolving the required amount of HBP in methanol. A known volume of stock solution of HBP was added to the 2 mL of buffer solution and the total volume was made to 4 mL with methanol. The solution was bubbled with nitrogen at a slow rate for 15 – 20 min before recording the curve. The SWV parameters were optimized and these optimized parameters were: initial (*E*): - 400 mV, final (*E*): - 1600 mV, square wave frequency: 15 Hz, sensitivity: 100 μA V^{-1}, square wave amplitude (E_{sw}): 20 mV and step (*E*): 4 mV. All the potentials are reported with respect to reference electrode at an ambient temperature of 25 ± 2^{o}C.

4.2.5 Characterization of product

For the characterization of product, about 10 – 12 mg of halobetasol was dissolved in 30 mL methanol and 30 mL of phosphate buffer of pH 7.2 (μ = 0.1 M). The solution was exhaustively electrolyzed by applying the potential ~ 70 mV more negative than the reduction peak potential of HBP using potentiostat. To remove the interference of oxygen, nitrogen bubbling was done

continuously at a slow rate. The progress of electrolysis was studied by withdrawing a sample from bulk electrolytic compartment and simultaneously recording cyclic voltammograms and UV spectra at time intervals of 15 min. When the absorption peak in the spectra completely disappeared (~ after 24 h), the exhaustively electrolyzed solution was removed from the cell and lyophilized. The material received after lyophilization, was extracted with methanol and the colorless dried material obtained was used for further characterization.

4.2.6 Analytical procedure

Halobetasol propionate is a trihalogenated agent and is available as 0.05 % ointment and cream preparation. Each 1 g of halobetasol cream generally contains 0.5 mg g^{-1} of HBP. In order to detect HBP in cream and ointment a known amount (5 g) of cream was weighed and dissolved in 25 mL of methanol and water in the ratio of 1: 4 followed by heating it at water bath until cream melts. After allowing the residue to settle, the hot solution was filtered and the extract was used for further experiments.

4.3 RESULTS AND DISCUSSION

4.3.1 Cyclic voltammetry

CV is one of the extensively used techniques providing the considerable information about the reversibility of the redox reaction. To accomplish this purpose, the electrochemical response of a solution of 0.5 mM of HBP was recorded by initiating the sweep in positive and negative directions. A well defined reduction peak was noticed when the sweep was initiated in the negative direction. No other peak was noticed in the voltammogram. Thus, HBP is irreversibly reduced giving the single reduction peak at ~ -1295 mV at SWCNT modified EPPGE which is shifted to more negative potential (~ -1335 mV) with a mark decrease in peak current at the bare EPPGE. These results clearly reveal that modification of electrode by SWCNT catalyze the reduction of HBP occurring at the surface of working electrode. A typical cyclic voltammogram of HBP is presented in **Fig. 4.2**.

To establish the nature of electrode reaction, scan rate studies were performed in the range of 10 – 350 mV s^{-1}. Scan rate > 350 mV s^{-1} could not be used as the peak changed to broad bump. Peak current due to the reduction of HBP was found to increase with scan rate and the dependence of peak current on scan rate can be expressed by following relation:

$i_p / v^{1/2} = 0.576 \log v - 0.317$

with a correlation coefficient of 0.97, where v is scan rate in mV s^{-1}. The linearity of plot of $i_p / v^{1/2}$ and log v as depicted in inset of Fig. 4.2 clearly indicated that the electrode reaction is governed by adsorption phenomenon [25, 26].

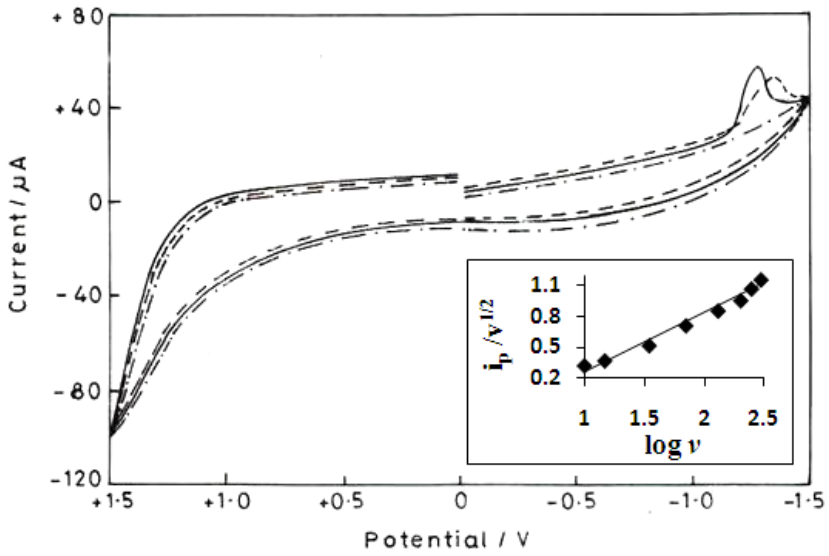


Figure 4.2: Cyclic voltammograms obtained for blank phosphate buffers at SWCNT/EPPGE (–·–·–·) and 0.5 mM halobetasol (in 50% methanol) at pH 7.2 using bare EPPGE (- - - -) and SWCNT/EPPGE (—) at 30 mV s^{-1}. Inset: variation of peak current with scan rate.

4.3.2 Square wave voltammetry

SWVs of 0.5 mM of HBP were recorded at bare and SWCNT/EPPGE. **Fig. 4.3** illustrates the voltammograms of HBP at bare and modified EPPGE at pH 7.2. Reduction of HBP occurs at lesser potential (~ -1265 mV) with enhancement of peak current at modified electrode in comparison to bare electrode. Appearance of reduction peak at less negative potential (~ -60 mV) with increament of peak current is sign of catalytic behavior of SWCNT/EPPGE towards HBP reduction.

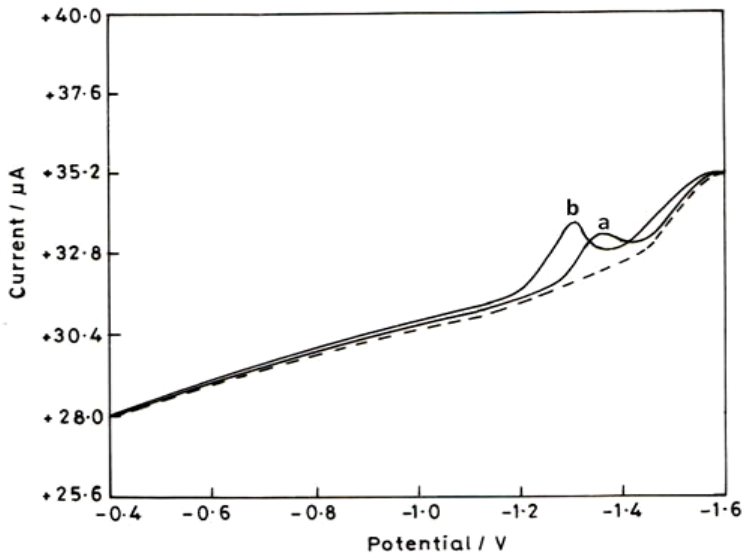


Figure 4.3: Comparison of SWVs of blank phosphate buffer at SWCNT/EPPGE (---) and 0.5 mM halobetasol (in 50% methanol) using (a) bare EPPGE and (b) SWCNT/EPPGE using pH 7.2.

4.3.2.1 Effect of pH

The electrochemical response of electrode is varied while changing the pH of supporting electrolytes. The impact of pH on reduction peak potential of HBP (0.5 mM) was monitored in the pH range 2.3 – 9.9 by using the bare and modified EPPGE. Reduction peak potential of HBP was found to be dependent and shifted to more negative potential with the increase in pH as shown in **Fig. 4.4 a**. The dependence of the E_p of reduction peak on pH at bare and nanotubes modified EPPGE can be represented by the relations:

$-E_p$ / mV = 56.30 pH + 884.7 at bare EPPGE

$-E_p$ / mV = 56.75 pH + 838.2 at modified EPPGE

having correlation coefficients of 0.996 and 0.995, respectively. The observed value of slope of the plots suggested that equal number of electrons and protons are participating [27] in electrochemical reduction of HBP. As physiological pH is close to 7.2; therefore, this pH was used for further studies.

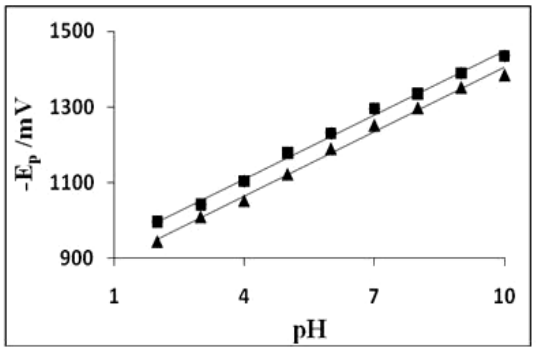


Fig. 4a

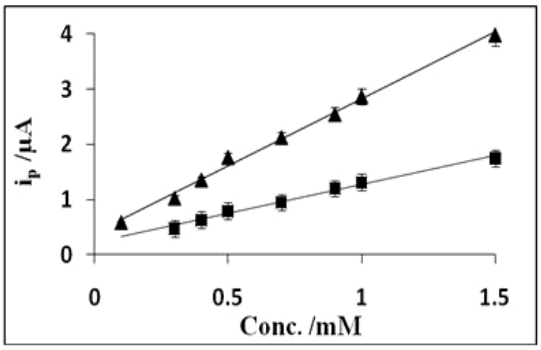


Fig. 4b

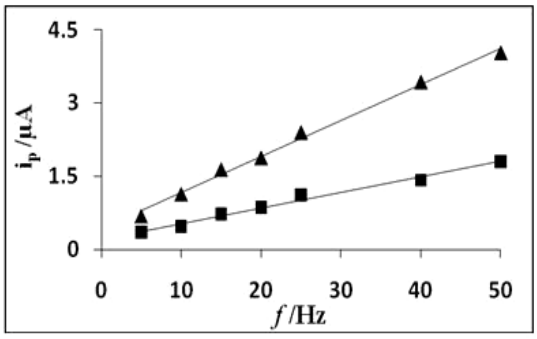


Fig. 4c

Figure 4.4: (a) Effect of pH on E_p at bare EPPGE (■) and SWCNT/EPPGE (▲), (b) Calibration plots at bare EPPGE (■) and SWCNT/EPPGE (▲) and (c) Variation of i_p with f at bare EPPGE (■) and SWCNT/EPPGE (▲).

4.3.2.2 Effect of concentration

The peak current of HBP increased with increasing concentration as shown in **Fig. 4.5.** The dependence of reduction peak current on increasing the concentration of HBP at bare and nanotubes modified edge plane pyrolytic graphite electrode is shown in **Fig. 4.4 b**. Cathodic peak current of HBP increases linearly in the concentration range of 0.02 mM to 1 mM. The dependence of reduction peak current on the concentration of HBP can be represented by equations:

i_p (µA) = 1.053 C + 0.216 at bare EPPGE

i_p (µA) = 2.432 C + 0.384 at modified EPPGE

having correlation coefficient of 0.990 and 0.994, respectively, where the term C represents milimolar concentration of HBP. The slope of the calibration plots corresponds to the sensitivity

1.053 µA mM^{-1} and 2.432 µA mM^{-1} at bare and modified EPPGE, respectively. The limits of detection for bare and nanotubes-modified electrode were found to be 33 µM and 10 µM, respectively, indicating the catalytic behavior of modified electrode towards the reduction of HBP.

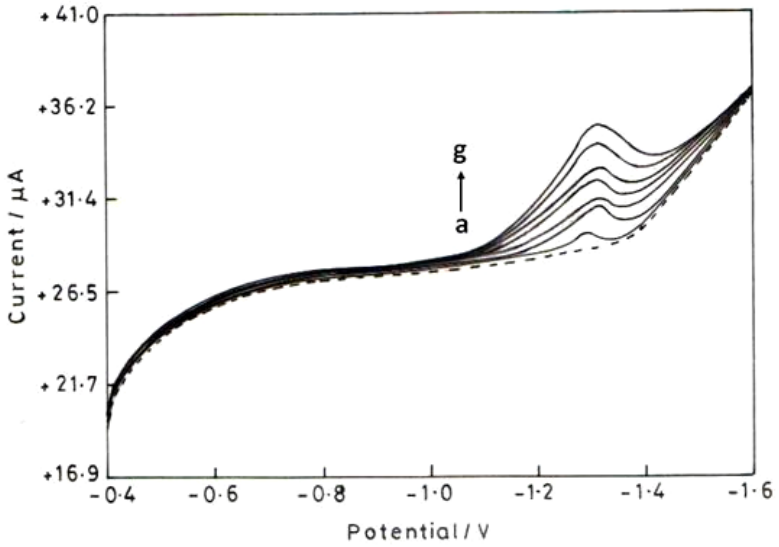


Figure 4.5: Observed SWVs for (i) blank phosphate buffer (background) (---) and (ii) increasing concentration of halobetasol (in 50% methanol). Curves were recorded at (a)=0.1; (b)=0.3; (c)=0.5; (d)=0.7; (e)=0.9; (f)=1.0 and (g)=1.5 mM concentrations using SWCNT/EPPGE in phosphate buffer of pH 7.2.

4.3.2.3 Effect of square wave frequency

The effect of square wave frequency on peak current of HBP was monitored in the frequency range of 5 – 50 Hz at pH 7.2. Studies at square wave frequency greater than 50 Hz could not be carried out because the reduction peak merged with the background. The peak current of 0.5 mM halobetasol propionate shows a linear increase with increase in square wave frequency (*f*) as shown in **Fig. 4.4 c**, suggesting that the electrode process is adsorption controlled [28]. The linear relationship between peak current and square wave frequency can be expressed by the relations:

i_p (µA) = 0.031 *f* (Hz) + 0.212 at bare EPPGE

i_p (µA) = 0.073 *f* (Hz) + 0.420 at modified EPPGE

with correlation coefficients of 0.987 and 0.994 for bare and modified EPPGE respectively.

4.3.3 Analytical utility of proposed method in pharmaceutical preparations

Prior to examine the applicability of the proposed method for the determination of HBP, the method was applied to the analysis of drug in various samples of pharmaceutical formulations. Different cream and ointments were analyzed for HBP concentration at nanotubes modified EPPGE in the phosphate buffer media of pH 7.2. By using the procedure mentioned as above, the extract of the filtered samples was further diluted by phosphate buffer of pH 7.2 so that the concentration of HBP lies in the range of calibration plot. Square wave voltammograms were then recorded and concentration of HBP was determined using calibration plot. The results obtained for HBP concentration are summarized in **Table 4.1**. The content for all assayed cream samples falls within the claimed amount, fulfilling the criteria of acceptance set according to the USP23 Uniformity of the Dosage Units [29]. Moreover, reference (labeled values) and observed amounts were compared. The calculated value obtained from student t-test is 0.21 for halobetasol cream samples (Table 1) at 95% confidence level indicates that there is no significant difference between the precision of claimed and observed amount.

Table 4.1: A comparison of observed and reported HBP concentration in pharmaceutical formulations using SWCNT/EPPGE.

Cream Sample	Reference amount (mg)	Observed amount (mg)	Error (%)
Halovate	0.199	0.195	-2.01
Halox	0.199	0.200	0.50
Halobet-S	0.199	0.194	-2.51

The accuracy of the proposed method was evaluated by its recovery during spiked experiments (the addition of known amounts of pure drug to pre-analysed formulations of halobetasol). For this purpose extract of filtered samples of HBP creams and ointments were diluted two times with phosphate buffer. A typical square wave voltammogram of sample 1 at SWCNT modified EPPGE is shown in **Fig. 4.6**. A well-defined peak was noticed at peak potential of ~ -1267 mV due to the reduction of HBP. The sample was then spiked with known concentration of standard solution of HBP. From voltammogram of Fig. 4.6, it can be clearly seen that peak current of reduction peak having E_p ~ -1267 mV increases significantly with addition of

HBP, thereby confirming that it corresponds to the reduction of HBP. The concentration of HBP was determined using the regression equation keeping in consideration the dilution factor.

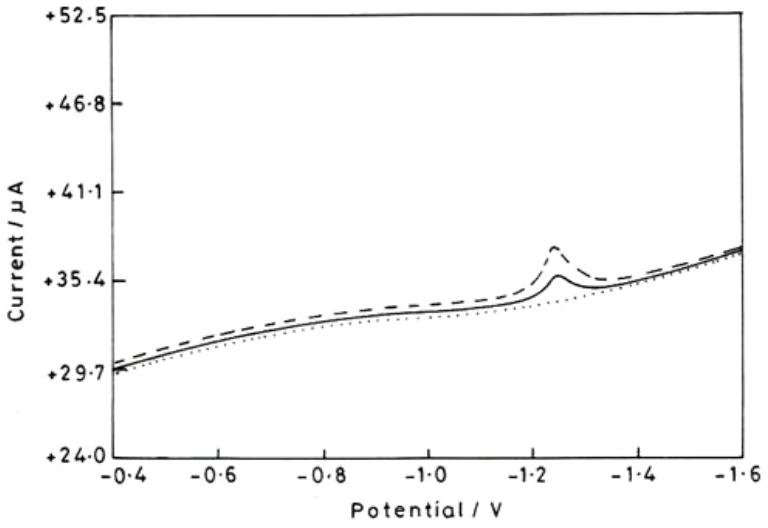


Figure 4.6: Square wave voltammograms observed for blank solution (background) (…..), halobetasol cream sample 1 (—) and sample 1 spiked with standard halobetasol (---), using SWCNT/EPPGE at pH 7.2.

The results obtained for different pharmaceutical samples, before and after the spiking, are tabulated in **Table 4.2** and clearly indicate that the developed protocol can be easily used for the determination of HBP in pharmaceutical samples. In order to detect interactions of excipients viz; KCl, NaCl and petroleum jelly as cream base, determination was carried out in their presence. As all these compounds are not reducible, they did not cause interference upto ~ 1000 times concentration. The standard addition technique was used to the same preparations, which were analyzed by the calibration straight line. These results indicate the validity of developed method for the quantitative assay of halobetasol in commercial samples. The R.S.D, Bias, average recovery and R.S.D of recovery were found as < 3.1 %, -1.34 % (Table 4.1), 100.85 % and 2.64 % (Table 4.2), respectively. The mean percentage recovery showed no significant excipients interference, so the procedure was able to determine halobetasol in the presence of excipients and thus it can be considered specific with reliable analysis.

Table 4.2: Concentration of HBP observed in pharmaceutical samples at SWCNT/EPPGE using standard addition method.

	Amount spiked (mM)	Amount detected (mM)	Recovery (%)
Sample 1	0.0	0.42	—
	0.1	0.54	103.85
	0.5	1.00	98.04
	1.0	2.05	101.48
Sample 2	0.0	0.41	—
	0.1	0.50	98.04
	0.5	1.04	102.97
	1.0	2.03	100.95

4.3.4 Stability and reproducibility of the modified electrode

Stability of SWCNT-modified electrodes for the determination of HBP was examined by measuring the current responses at fixed concentration of HBP at pH 7.2. The modified electrode was used daily and stored in air. The modified electrode showed a deviation in peak current of HBP by 3.34 % after a single day, while after a week modified electrode showed a relative standard deviation of 4.27 %. This suggests that modified electrode have sufficiently good stability.

The inter- and intra-day reproducibility of the proposed sensor was also evaluated. Deviation in current responses was calculated by using at least three replicate measurements of recorded voltammograms. Experimental results revealed that a R.S.D. of 0.76 % and 1.24 % was obtained while checking the intra-day and inter-day reproducibility respectively, of the CNT-modified EPPGE. Only minimal decrease in current responses is attributed to the excellent stability of the modified electrode.

4.3.5 Product characterization

The UV spectral changes of HBP during electroreduction were recorded in the region 200 – 400 nm at pH 7.2. Halobetasol propionate exhibits a characteristic UV absorption at λ_{max} of 237 nm. With the progress of electrolysis the absorbance at λ_{max} decreased and no new absorption maximum was noticed as shown in **Fig. 4.7**. This indicates that the product obtained from electrochemical reduction of HBP does not absorb in the region 200 – 400 nm. The GC-MS of electrolyzed product of HBP exhibited a prominent peak at R_t ~ 11.20 min having molar mass of 488 (MH^+). As molar mass of HBP is 485; it increases by 2 amu after reduction and indicates that the reduction of HBP occur in a $2e^-$, $2H^+$ process.

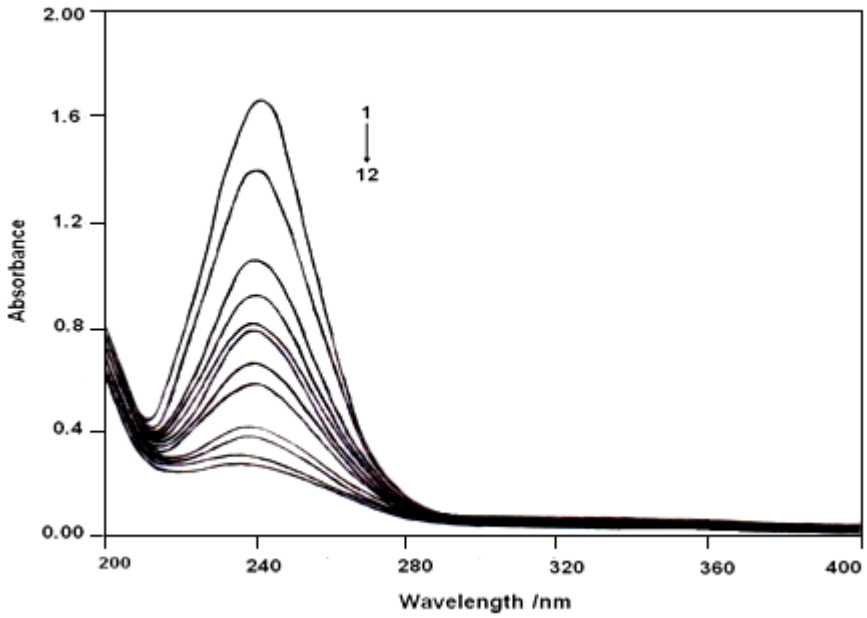


Figure 4.7: Observed UV spectral changes during electroreduction of halobetasol at - 1.3 V vs Ag/AgCl. The curves were recorded at (1) 0, (2) 15, (3) 30, (4) 60, (5) 120, (6)180, (7) 300, (8) 480, (9) 600, (10) 900, (11) 1200 and (12) 1440 min of reduction.

HBP contains two spatially separated chromophores, i.e. cyclohexadienone moiety in ring A and carbonyl group at C_{20}. Therefore, reduction may occur at any or both carbonyl groups. To confirm the reduction site of HBP, FT-IR spectrum of HBP was recorded. The IR characteristic absorption bands were observed at 3439 (O-H str.), 2926, 2859 (C-H str.), 1718 (C=O str. in ester), 1664 (cyclic C=O str.), 1622 (acyclic C=O str.), 1450, 1385 (C-H def.) and 1076 cm^{-1} (C-O str.). The characteristic absorption near 1664 cm^{-1} due to cyclic C=O str. did not appear in FT-IR

spectrum of the product, rather an extra absorption band near 3133 cm^{-1} (O-H str.) was observed **(Fig. 4.8)**. Thus, it is concluded that the site of reduction in HBP is at C-3 position.

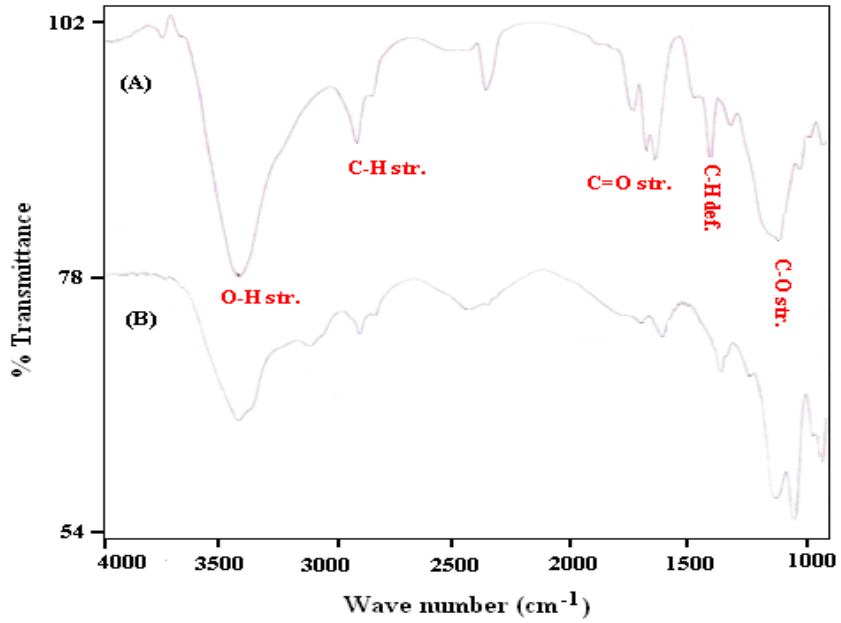


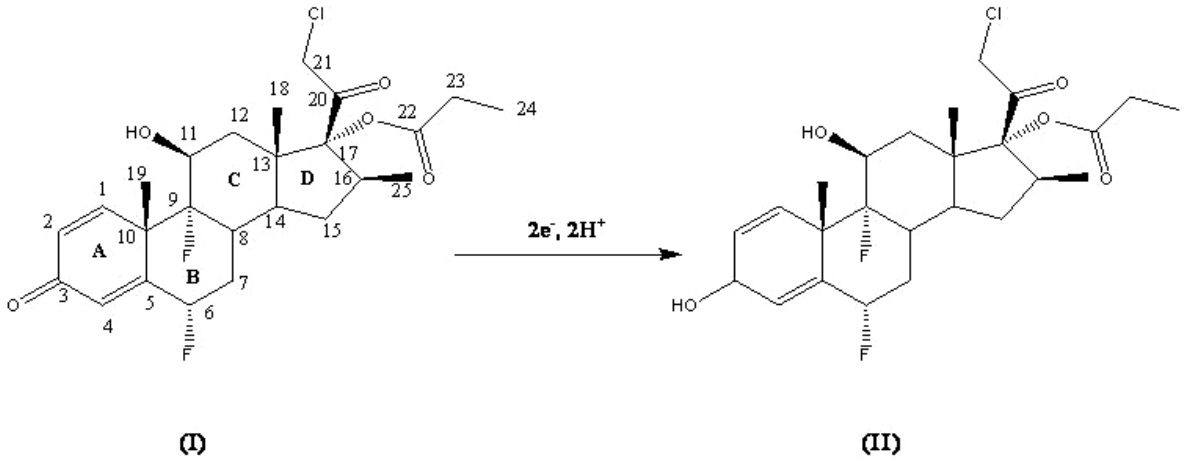
Figure 4.8: FT-IR spectra observed for HBP (A) and its reduction product (B).

To further confirm the site of reduction, ^{1}H-NMR spectra were also recorded. The NMR spectrum of HBP indicated the signals essentially similar to the ones reported in literature [12]. The signals corresponding to rings B, C and D were found to remain unaffected while signals due to the ring A were strongly modified in the product. All chemical values of protons corresponding to rings B, C and D were similar in the reactant and product, however, in the product an extra peak having chemical shift of 4.39 ppm (bs) was observed. This signal is assigned to the reduction of C-3 carbonyl group. Three of the olephenic -CH (ring A) were conserved but having lower δ value than observed in the reactant because >C=O was converted into CH-OH and deshielding effect of >C=O had been removed in the product. The details of all the NMR signals observed are shown in **Table 4.3**. Above results clearly indicate that conjugated carbonyl group in ring A of HBP undergoes reduction and carbonyl group at position 20 remains unaffected during electrochemical reduction of HBP. It has also been reported earlier that conjugated carbonyl group undergoes easier reduction than the isolated one [30, 31]. Thus, the reduction in HBP occurs at C-3 position where keto group is converted to hydroxyl group by $2e^{-}$, $2H^{+}$ process as shown in **Scheme 1**.

Table 4.3: A comparison of ^{1}H-NMR signals observed for halobetasol (I) and product (II).

No*	I	II
1	7.15 (d, 1H)	6.43 (d, 1H)
2	6.34 (dd, 1H)	5.89
3	-	4.39 (bs)
4	6.42 (bs, 1H)	5.31
6	5.40 (ddd, 1H)	5.61 (ddd, 1H)
7	2.31, 1.65	2.26, 1.58
8	2.49	2.53
11	4.37	4.12
12	2.26, 1.43	2.34, 1.50
14	2.03	2.11
15	1.53, 1.20	1.51, 1.15
16	2.18 (t, 1H)	2.28 (t, 1H)
18	1.06 (s, 3H)	1.02 (s, 3H)
19	1.65 (s, 3H)	1.59 (s, 3H)
21	4.18	4.01
23	2.29 (q, 2H)	2.30 (q, 2H)
24	1.14 (t, 3H)	1.12 (t, 3H)
25	1.15 (d, 3H)	1.06 (d, 3H)

*Scheme 1 for numbering



(I) (II)

Scheme 1: Tentative mechanism proposed for the reduction of HBP.

4.4 CONCLUSIONS

The surface modification of EPPGE by single-walled CNT improves its electrochemical properties by enhnacing the peak current and shifting the peak potential of HBP to the less negative values. The modified electrode presents many enticing advantages towards the voltammetric detection of HBP such as improved peak shape, high sensitivity and low detection limit. Modified electrode also exhibits immense stability and excellent reproducibility along with high accuracy which makes it appropriate for the determination HBP in clinical preparations. The large surface area of CNT embedded metal impurities present in nanotubes and edge-plane-like defects, which are found at the open endes of nanotubes, have been assigned as the origin of electro catalytic properties of nanotubes [32, 33]. The purity of carbon nanotubes may affect the peak potential and peak current as observed earlier [33-35] and thus sample to sample change may cause some variation. However, such variations for CNT for purity > 98% will be minimal. The method eliminates the need for time consuming and tedious derivatization and extraction steps prior to analysis. Statistical calculations including student's t test, RSD %, Bias % and recovery % clearly indicate that the analysis of medicinal samples using proposed method has excellent accuracy as the detected content was in good agreement with the labeled (reference) values. The characterization of the product indicates that the reduction takes place at carbonyl group present at position 3 and not at 20. It is necessary to mention that very few attempts [1, 36] have been made

to determine HBP. Spectrophotometric determination using charge transfer complexes has reported a detection limit of 20 μg mL^{-1}, which is higher than observed in the present method. It is thus concluded that the proposed protocol is a good approach for sensitive determination of HBP due to its simplicity, selectivity and relatively short analysis time.

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Chapter 5

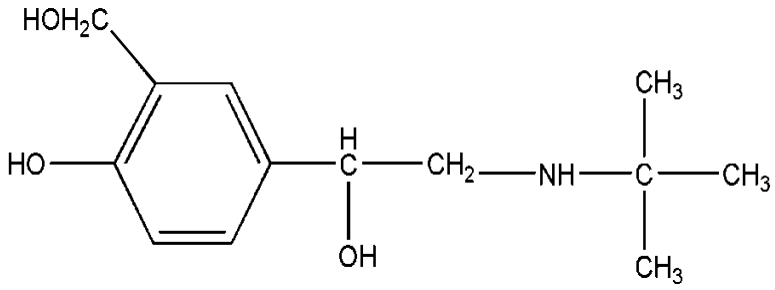
SWCNT based Electrochemical Sensor for Salbutamol; a Doping Agent

5.1 INTRODUCTION

β_2–agonists are effective bronchodilators and normally used in symptomatic treatment of asthma and chronic bronchitis [1-3]. Salbutamol (α^1-[(tert-butylamino) methyl]-4-hydroxy-m-xylene-α, α'-diol) is a direct-acting β_2-agonist with beta-adrenergic activities employed as bronchodilator for the treatment of asthmatic disorders and chronic obstructive pulmonary diseases [4, 5]. It is also used to reduce premature labor in pregnancy [6]. Salbutamol is commonly administrated as pressurized metered dose inhaler and nebulized aerosol [7]. Salbutamol has been pharmacologically proven to be able to increase muscle protein, reduce total body fat and promote muscle growth; therefore, it is highly abused by athletes to improve muscular strength and in turn improve their performance in sports [8-10]. The list of prohibited substances in sports published by the WADA specifies that salbutamol can be used only by inhalation. Administration of salbutamol by oral or parenteral route and very large inhaled dose are forbidden due to strong adrenergic stimulation. The threshold concentration of 1000 ng mL^{-1} has been established to suspect for oral administration of salbutamol by the WADA rules [11, 12]. The concentration of salbutamol greater than 1000 ng mL^{-1} is considered as an adverse analytical finding of anabolic agent and constitutes a doping violation [13]. In view of extensive use and misuse of salbutamol in sports, it has been mandatory to analyze salbutamol concentration in pharmaceutical formulations as well as in biological fluids.

Several techniques have been explored for the determination of salbutamol to monitor the therapeutic use as well as to control illegal use including high performance liquid chromatography [14], capillary electrophoresis with UV detection [15], flow-injection analysis using spectrophotometric method [16], solid-phase extraction method [17], conductometric method [18] and electro generated chemiluminescence detection [19]. Although spectrophotometry and chromatography are the most commonly employed techniques but these involve many derivatization steps and an effective extraction purification approach prior to final analysis which is very time consuming and demand of expensive and heavy instrumentation. The voltammetric methods have been proven to be advantageous alternative way due to their simplicity, high sensitivity and rapidness [20, 21]. Hence, the determination of salbutamol at variety of electrodes has been attempted [22-27]. In many of these investigations, biological samples particularly urine has not been analyzed; hence, their usefulness could not be ascertained. In several others, the detection limit was observed much higher than expected to be present in urine or blood plasma. In

the last few years, our laboratory has been also trying to develop an efficient sensor for the determination of salbutamol [23]; however, at nanogold modified electrode, a detection limit of 0.30 μM is achieved. As the preparation of nanogold electrode is tedious and the size of nano particles depends on time for which ITO sheet is dipped in the solution for crystal growth [28], pyrolytic graphite modified with carbon nanotubes is used in the present studies. The aim of the present work is to develop a fast and sensitive voltammetric sensor for the direct determination of salbutamol in human body fluids particularly urine as it can easily detect the cases of doping at the site of competitive games. The EPPGE has been established as a useful substrate for detecting the lower concentration of biomolecules and drugs compared to other conventional electrode [29, 30]. Carbon nanotubes are expected to increase electrochemical performance of electrodes due to their excellent electrical conductivity, nanometer size and good chemical stability [31-35]. Therefore, the studies have been performed at SWCNT/EPPGE. Square wave voltammetry is one of the widely used techniques due to its higher sensitivity, simplicity and lower limit of detection for drugs and biomolecules [36, 37]; hence; it has been utilized for the sensitive sensing of salbutamol in body fluids and pharmaceutical preparations to monitor clinical and doping cases.



[Salbutamol]

5.2 EXPERIMENTAL

5.2.1 Instrumentation

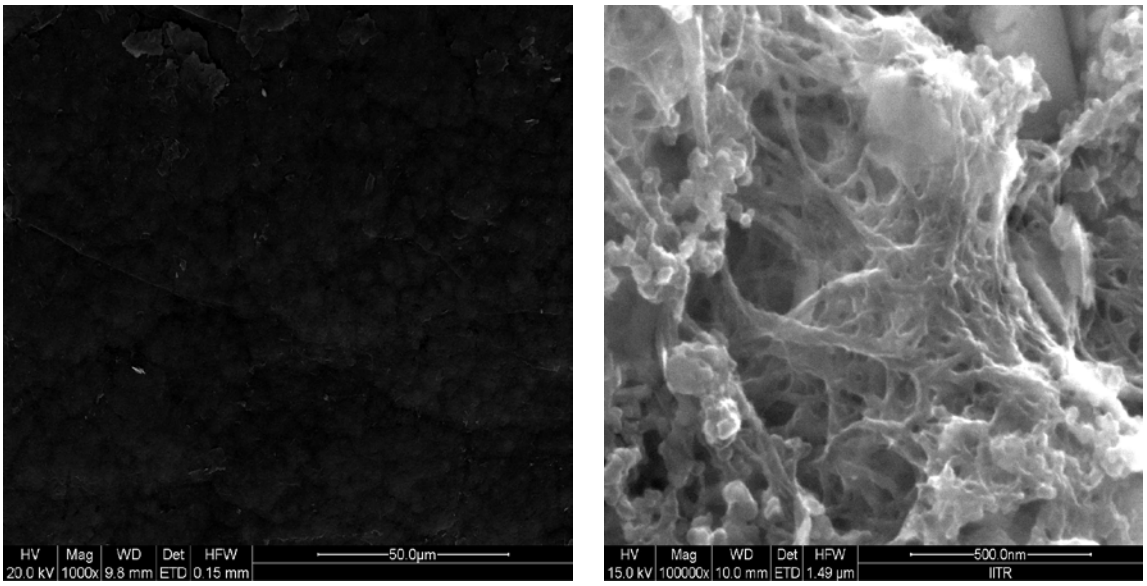
The voltammetric studies were carried out using BAS (West Lafayette, USA; CV-50W) voltammetric analyzer. The voltammetric cell used was a single compartment glass cell containing SWCNT/EPPGE as working electrode, a platinum wire as counter electrode and Ag/AgCl as reference electrode (3 M NaCl, model BAS MF-2052 RB-5B). The edge plane pyrolytic graphite piece was obtained from Pfizer Inc., New York, USA and the electrode was prepared as reported earlier in literature [38]. The pH of the buffer solutions was measured using digital pH meter (Model CP-901, Century India Ltd.). All potentials are reported with respect to Ag/AgCl reference electrode at an ambient temperature of 27 ± 2^{o}C.

5.2.2 Chemicals and reagents

Salbutamol sulphate in powdered form was obtained as a gift sample from Vamsi labs Ltd., Maharashtra, India. SWCNT of purity > 98 % was purchased from Bucky USA, Houston, TX, USA. Salbutamol-containing tablets of different companies were purchased from local market. Phosphate buffers (1 M) were prepared according to the method of Chiristian and Purdy [39]. All chemicals used were of analytical grade and were purchased from Merck. Double distilled water was used throught the experiments.

5.2.3 Preparation of SWCNT/EPPGE

Prior to modification, the surface of EPPGE was rubbed on an emery paper followed by cleaning it with double-distilled water and softly touching with tissue paper. Firstly, different concentrations of nanotubes in N, N-dimethyl formamide were prepared. Then, 0.5 mg mL^{-1} was selected as an optimum based on the optimum current response of fixed concentration of salbutamol. A 0.5 mg mL^{-1} suspension of SWCNT was prepared by dispersing 0.5 mg SWCNT in 1.0 mL DMF by ultrasonic agitation. A known volume (40 µL) of this suspension was coated onto the surface of the bare EPPGE and the solvent was allowed to evaporate at room temperature. The modified electrode was now ready for use. The surface morphology of the bare and modified electrode was characterized by recording FE-SEM using Quanta 200 FE-SEM instrument. A comparison of FE-SEM images of the bare and SWCNT-modified electrodes is presented in **Fig. 5.1** and clearly indicates the deposition of SWCNT on the surface of electrode.



(a) **(b)**

Figure 5.1: Typical FE-SEM images of (a) bare and (b) SWCNT/EPPGE.

5.2.4 Analytical Procedure

Stock solution of salbutamol (1 mM) was prepared by dissolving the required amount of compound in double-distilled water. A known amount of stock solution was added to 2.0 mL of phosphate buffer. The total volume was made 4.0 mL with double distilled water. Square wave voltammograms were then recorded at the optimized parameters: initial potential: 0 mV, final potential: 1200 mV, square wave frequency: 15 Hz, square wave amplitude: 20 mV and step potential: 4 mV. Urine sample of asthma patient (male: 30 years, 45 kg) undergoing treatment with salbutamol from 8 months and blood plasma samples of healthy volunteers (female: 25 years, 50 kg; male: 30 years, 55 kg) were obtained from institute hospital of IIT Roorkee. Blood sample was ultra centrifuged at a speed of 1000 rpm for 5 min and supernatant blood plasma was used for the determination of salbutamol. Phosphate buffer of pH 7.2 was used for 2 and 4 times dilution of urine and blood samples, respectively.

5.3 RESULTS AND DISCUSSION

5.3.1 Comparison of bare and modified electrode

Electrochemical properties of salbutamol were demonstrated by using square wave voltammetry at bare and SWCNT-modified EPPGE in phosphate buffer of pH 7.2. **Fig. 5.2** clearly

indicates that on scanning the potential from 0 to 1200 mV, oxidation peak was noticed at ~ 660 mV (peak a) using the bare electrode while for the modified electrode oxidation peak appeared at ~ 600 mV (peak b) having marked increment in current value as compared to the bare electrode for 2000 ng mL^{-1} salbutamol. The significant improvement in peak current with decreasing peak potential clearly demonstrates that SWCNT act as an efficient electron mediator for the oxidation of salbutamol. The edge-plane-like defects which are present at the open ends of nanotubes and embedded metal impurities in CNT samples are important reasons, responsible for electrocatalytic properties of nanotubes [40, 41]. The modified electrode acts as a better substrate for voltammetric oxidation of salbutamol; therefore, further detailed studies were carried out at SWCNT-modified EPPGE.

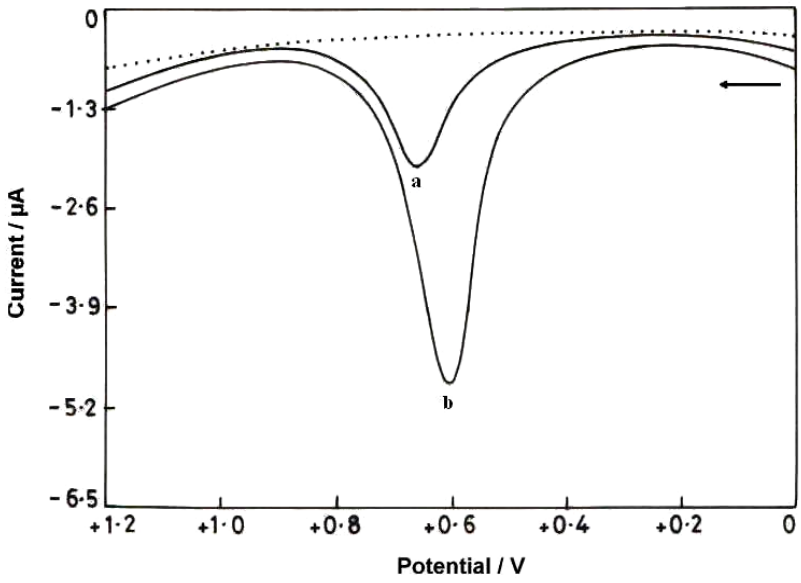


Figure 5.2: Comparison of square wave voltammograms for 2000 ng mL^{-1} of salbutamol at bare electrode (peak a) and SWCNT-modified electrode (peak b) at pH 7.2 and background at SWCNT/EPPGE is shown by dotted line.

5.3.2 Cyclic voltammetry

Cyclic Voltammetry is one of the most widely used techniques which provide considerable information about the electrode reaction. Electrochemical response of salbutamol was determined by recording the cyclic voltammogram of 5000 ng mL^{-1} salbutamol at SWCNT-modified EPPGE in phosphate buffer of pH 7.2 at scan rate of 20 mV s^{-1}. **Fig. 3** shows that a well-defined anodic

peak is observed with peak potential of ~ 657 mV for salbutamol oxidation at SWCNT/EPPGE. Absence of any peak in the reverse sweep clearly indicates that salbutamol oxidized irreversibly at SWCNT/EPPGE.

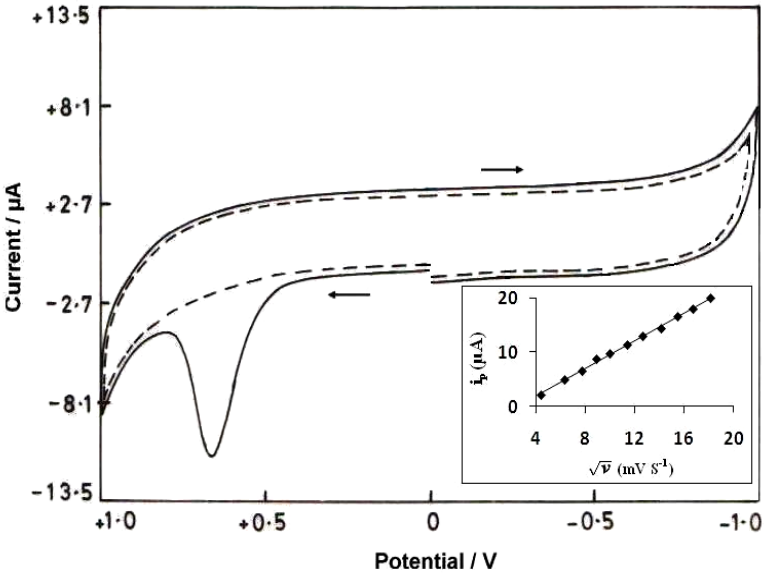


Figure 5.3: Cyclic voltammogram for 5000 ng mL^{-1} of salbutamol at SWCNT/EPPGE (—) and background at SWCNT-modified EPPGE (…...) at pH 7.2 at scan rate of 20 mV s^{-1}. Inset: effect of scan rate on peak current of sabutamol.

Then, to analyze the nature of electrode reaction, cyclic voltammograms of 2000 ng mL^{-1} salbutamol were recorded at different scan rates in the range from 20 to 330 mV s^{-1} as shown in inset of Fig. 5.3. It was found that peak current increases linearly with increase in scan rate and the plot of i_p versus $v^{1/2}$ clearly indicated that the reaction occurred at the surface of modified electrode is governed by the diffusion process [42, 43]. The dependence of peak current on scan rate can be expressed by the relation:

$$i_p\ (\mu A) = 1.273\ v^{1/2} - 3.223$$

where v is scan rate (mV s^{-1}) having a correlation coefficient of 0.997. Since SWV is more sensitive than other voltammetric techniques and has advantage of suppressing the background current; hence, further studies for the determination of salbutamol in real samples were performed by square wave voltammetry.

5.3.3 Effect of pH

The pH of the supporting electrolyte is one of the variables that strongly affect the redox reaction of analytes. Hence, it is usually important to investigate the effect of pH on electrochemical system. In order to optimize pH, the effect of pH on the oxidation of salbutamol was studied in the range of 2.3 to 9.9 using SWCNT/EPPGE. The peak potential (E_p) of salbutamol was found to shift towards the less positive potential with increase in pH as shown in **Fig. 5.4**. The linear dependence of peak potential on pH can be expressed by following relations:

E_p (mV) vs. Ag / AgCl = - 51.52 pH + 980.9 at SWCNT/EPPGE

E_p (mV) vs. Ag / AgCl = - 52.05 pH + 1041.1 at bare EPPGE

having correlation coefficients of 0.995 and 0.992, respectively. The observed slope of ~ 52 mV pH^{-1} clearly indicates that equal number of electron and protons are involved in the electrode reaction [44]. It was found that at pH 6.0 peak current was comparatively higher than pH 7.0 for salbutamol solution; however, study was performed in neutral media (pH 7.20) owing to the fact that pH of human body fluids is almost equal to 7.00 and determination of salbutamol in human body fluids was the main aim of proposed work.

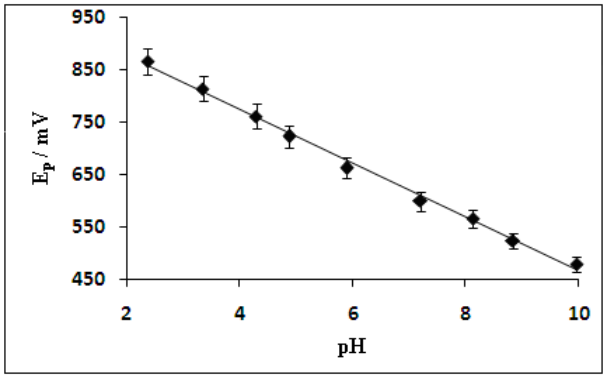


Figure 5.4: Dependence of pH on peak potential of salbutamol at SWCNT/EPPGE.

5.3.4 Study of the linear Range, sensitivity and detection Limit

Square wave voltammograms of different concentration of salbutamol were recorded in order to plot calibration curves using bare and modified electrodes. A systematic increase in peak current is observed with increase in concentration of salbutamol at electrodes surface. The peak current versus concentration plots present a good linearity for bare and modified electrodes in the

concentration range of 500 – 2500 ng mL^{-1} and 50 – 2500 ng mL^{-1}, respectively, as depicted in **Fig. 5.5**. Linear relations between peak current and concentration of salbutamol can be expressed at both the electrodes by the following equations:

i_p (nA) = 2.147 C + 0.445 at SWCNT/EPPGE

i_p (nA) = 0.830 C + 2.125 at bare EPPGE

where C is the concentration (ng mL^{-1}) of salbutamol having correlation coefficients of 0.996 and 0.995, respectively.

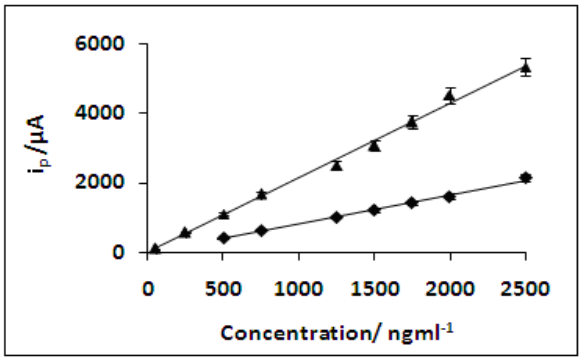


Figure 5.5: Calibration plot of salbutamol at bare (■) and SWCNT modified EPPGE (▲) at pH 7.2.

Fig. 5.6 illustrates a series of square wave voltammograms obtained for sabutamol at different concentrations in 1 M phosphate buffer of pH 7.2 using SWCNT/EPPGE. The detection limit for modified electrodes was calculated by using the formula $3\sigma / b$, where σ is the standard deviation and b is the slope of calibration curve and found to be 4.31 ng mL^{-1}.

5.3.5 Stability and reproducibility of SWCNT/EPPGE

Reproducibility and stability are two important parameters of an electrode for the selective and sensitive quantitative determination. Stability of modified electrode was examined by measuring the current response at fixed concentration of salbutamol over a period of 15 days. Electrode was used daily and stored in air. The experimental results show that the current responses deviate intraday by 1.28 % and interday by 1.90 %, suggesting thereby that SWCNT-modified EPPGE possesses good stability for the determination of salbutamol.

The reproducibility of modified electrode was evaluated by the repetitive measurements (n = 6) of salbutamol at a fixed concentration of 10 μM. The corresponding relative standard deviation of 0.4 % confirms that results are satisfactorily reproducible. In order to examine intraday (repeatability) and interday (reproducibility) response, SWVs were recorded for fixed concentration of salbutamol (10 μM) using SWCNT/EPPGE. The experimental results show that the current responses deviate intraday by 1.94 % and interday by 2.40 %, suggesting thereby that SWCNT/EPPGE possesses adequate reproducibility for the determination of salbutamol. Intra-electrode reproducibility is also an important parameter; hence, to examine electrode-to-electrode variation response, four pyrolytic graphite electrodes (1 × 1 × 3 mm^3) were casted with 40 μL of SWCNT suspension. It was observed that these electrodes show a variation of ± 2.9% in peak current of 10 μM salbutamol. Thus, it is concluded that the electrode to electrode variation is nonsignificant. Thus, the SWCNT-modified EPPGE exhibits a good reproducibility and stability for the determination of salbutamol and therefore the proposed sensor is also recommended for the determination of similar drugs and biomolecules with good sensitivity and low detection limit.

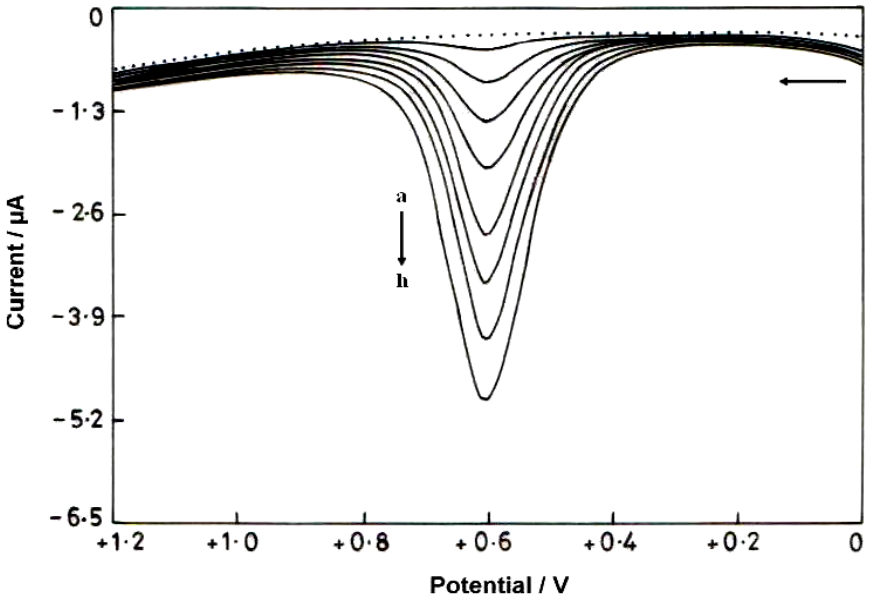


Figure 5.6: Square wave voltammograms of various concentrations of salbutamol at SWCNT-modified EPPGE in 1 M phosphate buffer (pH 7.2); [salbutamol]: a=50, b=250, c=500, d=750, e=1250, f=1500, g=1750 and h=2000 ng mL^{-1}.

5.3.6 Analytical utility

5.3.6.1 Real sample analysis

In order to ascertain the analytical utility of the proposed method, attempts have been made to analyze the urine sample of an asthma patient undergoing treatment with salbutamol for last 8 months. Urine sample was collected after oral administration of 2 mg salbutamol tablet (Salbetol-2). Prior to analysis, urine sample was diluted 2 times with phosphate buffer of pH 7.2 in order to minimize interference of matrix. Square wave voltammograms of urine sample of patient and spiked with known amount of salbutamol were then recorded under optimized parameters using SWCNT-modified EPPGE. **Fig. 5.7** clearly shows that a well-defined oxidation peak was observed at ~ 600 mV (peak b) in urine sample of the patient.

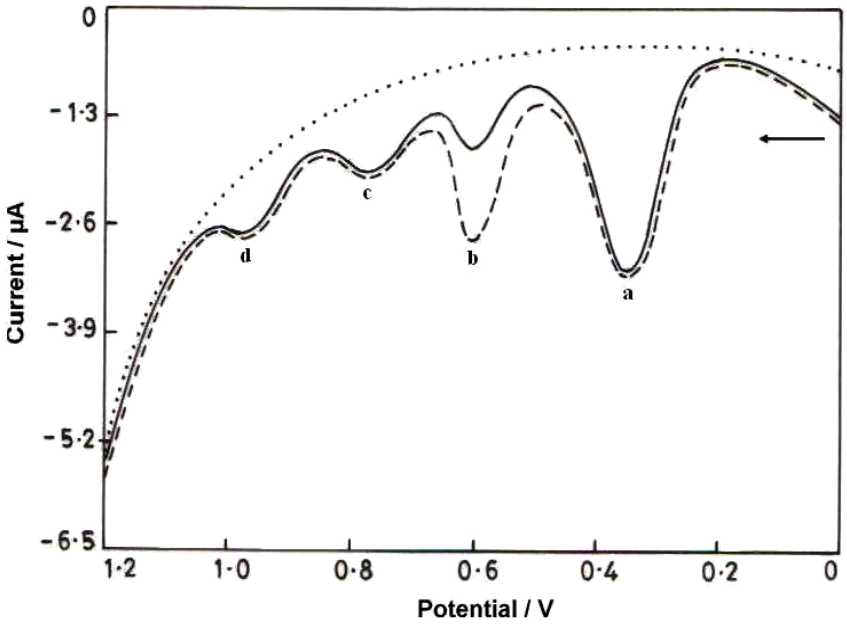


Figure 5.7: SWVs for urine sample of asthma patient (—) and urine sample of patient spiked with salbutamol (---) using SWCNT/EPPGE. The background at modified electrode is shown as (…).

In order to confirm that this peak is due to the excreted salbutamol in urine, a known amount of authentic salbutamol was spiked in diluted urine sample of patient. It was observed that the peak current of peak at ~ 600 mV increased on spiking of salbutamol, indicating thereby that the peak at E_p ~ 600 is due to the oxidation of salbutamol excreted in urine sample of patient. Some additional peaks were also observed in urine sample at ~ 300 (a), ~ 780 (c) and ~ 980 mV

(d) and were likely to be due to the oxidation of major urinary metabolites viz. uric acid, xanthine and hypoxanthine, respectively, although no efforts had been made to analyze them. The concentration of salbutamol in patient's urine sample was calculated by using calibration plot and found to be 2.0 µM, which was further confirmed using standard addition method. The RSD for the determination was found to be less than 2.2 % for n = 5.

5.3.6.2 Pharmaceutical analysis

Proposed method was also used to analyze salbutamol-containing tablets to determine their salbutamol content using SWCNT-modified EPPGE. The salbutamol content was determined in five common medicinal tablets; Salbetol-2 (Verna industrial estats verna, FDC Ltd., Mfg. Lic No. 656), Asthalin-2 (Mfd. by Cipla Ltd.), Asthalin-4 (Mfd. by Cipla Ltd.), Ventorlin-8 (Mfd. by Themis laboratories Pvt. Ltd., Mfg. Lic No.; KD-638), Eto-Salbetol (Mfd. by Kare labs Pvt. Ltd., Mfg. Lic. No.; 314). Firstly, tablets were crushed and dissolved in double-distilled water. The solutions obtained by dissolution of tablets were subsequently diluted so that reported salbutamol concentration falls in the range of calibration plot. SWVs were then recorded under identical conditions which were used during plotting the calibration plot. Concentration of salbutamol determined by employing the proposed method in different pharmaceutical formulations is compared with labeled concentration of salbutamol and summarized in **Table 5.1**. The results show that salbutamol content for all pharmaceutical preparations fall within the claimed amount with error of ± 2.5% indicating the adequate accuracy of the proposed method.

Table 5.1: Determination of salbutamol in different pharmaceutical preparations using SWCNT/EPPGE.

Tablet	Amount labeled (mg)	Amount detected (mg)	Error %
Salbetol-2	2.00	2.05	+ 2.5 %
Asthalin-2	2.00	2.04	+ 2.0 %
Asthalin-4	4.00	3.90	- 2.5 %
Ventorlin-8	8.00	7.99	- 0.12 %
Eto-Salbetol	2.00	1.99	- 0.5 %

5.3.7 Recovery study

In order to examine the stability of salbutamol in human body fluids and the accuracy of the developed method, recovery experiments were carried out at SWCNT/EPPGE. SWVs of plasma samples taken from two healthy volunteers were recorded for this purpose. Recovery experiments were done by using standard addition method using SWCNT-modified EPPGE. The drug free plasma samples were spiked with known concentrations of standard solution of salbutamol followed by recording their voltammograms. The concentration of salbutamol was calculated by using regression equationand the results observed are tabulated in **Table 5.2**. The recoveries varied in the range from 98.00 % to 100.00 % with relative standard deviation of ± 3.2 %, indicating good accuracy of the proposed sensor and adequate stability of salbutamol in body fluids is also recommended.

Table 5.2: Recovery data for salbutamol determination in plasma samples of healthy volunteers at SWCNT-modified EPPGE.

Spiked amount (µM)	Detected amount (µM)	Recovery (%)
Sample 1		
0.50	0.50	100.00
1.00	0.99	99.00
1.50	1.49	99.33
Sample 2		
0.50	0.49	98.00
1.00	0.98	98.00
1.50	1.49	99.33

Table 5.3: A comparison of voltammetric response of SWCNT/EPPGE with earlier reported methods for the determination of salbutamol.

Sr.no.	Electrode	Concentration range (μM)	Limit of detection (μM)	Sensitivity (μA/μM)	Analytical utility Real samples	Ref.
1.	Pt electrode	100 – 1000	80.00	0.012	No	22
	GCE	20 – 1000	10.00	0.066	No	22
2.	NGITO	0.20 – 8.35	0.31	0.055	Yes	23
3.	GCE	0.80 – 80.00	0.20	—	No	24
4.	MWCNT/GCE	0.80 – 10.00	0.20	0.620	No	25
5.	C_{60}/GCE	0.42 – 8.35	0.17	0.048	Yes	26
6.	GN/GCE	5 – 90	0.10	—	No	27
7.	SWCNT/EPPGE	0.20 – 10.45	0.018	0.520	Yes	Proposed method

5.4 CONCLUSIONS

The results presented in this paper indicated that square wave voltammetry associated with the application of SWCNT/EPPGE serves as a fast and reliable tool for the analysis of salbutamol in biological system. SWCNT/EPPGE exhibits improved electrocatalytic properties with enhanced peak current and decreased peak potential as compared to bare EPPGE and several other electrodes. The ability to mediate fast electron transfer reaction with salbutamol in solution makes modified electrode an ideal candidate for its use in electrochemical experiments. The two important analytical parameters for efficient quantitative determination are sensitivity and detection limit. It was found that sensitivity of salbutamol determination was almost three times higher using modified electrode as compared to bare electrode and detection limit was also low enough at SWCNT/EPPGE. Findings of the proposed work prove that the above-described approach can be a desirable pathway for fabrication of sensors based on nanotubes systems. It was also found that detection limit, sensitivity and practical utility of the proposed method utilizing SWCNT/EPPGE are much better than earlier reported methods **(Table 5.3)**. The detection limit at

proposed electrode is almost ten and sixty times lower than reported recently at MWCNT/GCE and graphite nanosheet-modified electrodes, respectively [25, 27] and sensitivity is ten times higher than nanogold-modified ITO [23]. Real sample analysis is an important analytical utility of any sensor; however, no information regarding real sample analysis has been provided in recently reported method using MWCNT/GCE for salbutamol determination. Thus, it is reasonable to conclude that SWCNT/EPPGE is a better sensor for determination of salbutamol in comparison to nanogold-modified ITO or several other conventional electrodes reported earlier (Table 5.3). The proposed sensor showed a good linear range, low detection limit, good reproducibility, satisfactory recovery results and high stability making this system a promising example of electrochemical sensor and interesting alternative for quantification of salbutamol in human body fluids as well as in commercial preparations.

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Chapter 6

Determination of Important Analgesic Drugs using Bare EPPGE

6.1 INTRODUCTION

In earlier chapters, pyrolytic graphite electrode was modified with carbon nanotubes and used for the determination of biomolecules and drugs. However, unmodified PGE has also been used for the electroanalysis purposes due to its long term stability and strong adsorption capability. In view of these properties and to avoid surface modification procedure, which often requires stability and other studies, the edge plane of unmodified PGE is used for studying redox reactions of analgesics in this chapter. It is observed that unmodified PGE is superior over other simple electrode surfaces [1-4].

Analgesic drugs are mainly of two types; anti-inflammatory drugs and opioid drugs. Anti-inflammatory drugs are used for short term pain relief and for modest pain by reducing the local inflammatory responses, while opioid drugs can be used either for short term or long term pain relief and act on the brain. Analgesic drugs inhibit the synthesis of prostaglandins, which is the natural product of inflamed white blood cells and reduces its release in local tissues. These drugs act in different ways on peripheral and central nervous system and include acetaminophen and the aspirin like drugs or non-steroidal anti-inflammatory drugs (NSAIDs). NSAIDs are the most commonly employed first line drugs for the treatment of several diseases. In recent times, the use of NSAIDs for the treatment of pain and inflammation is ubiquitous [5].

The present chapter includes the determination of important analgesic drugs and for clearly explaining the results, this chapter is divided into two sections; first section deals with the investigation of diclofenac and second section describes the determination of two important analgesic drugs; aspirin and caffeine. Diclofenac (I) a well known representative of NSAIDs, used to treat several pathologies is chosen as the model drug in the present investigation. It is an extensively used drug with analgesic, antipyretic and anti-inflammatory properties [6]. It is widely prescribed in clinical medicines for the treatment of rheumatoid arthritis, osteoarthritis, non-articular rheumatism and sport injuries [7-9]. Diclofenac has been used to relieve the symptoms of diseases such as, ankylosing spondylitis and acute muscle pain conditions [10]. It is also used as an adjuvant in the treatment of chronic diseases such as glaucoma [11]. The therapeutic action of diclofenac is based on its ability to serve as a potent inhibitor of cyclooxygenase enzymes preventing the production of prostaglandins [12]. Diclofenac is also topically administrated in the form of a 1.16 % gel, which provides an effective short-term reduction in elbow pain and wrist extensor weakness associated with chronic lateral epicondylitis [13]. The use of diclofenac has

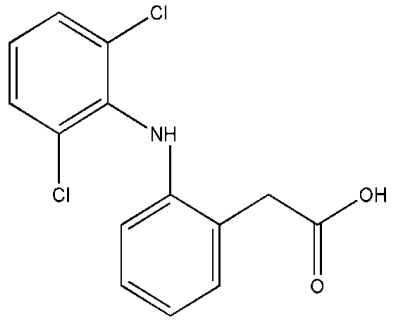
also been found to cause hematological toxicity, aplastic anemia, neutropenia, hepatitis, hemolytic anemia and thrombocytopenia [14]. Treatment with diclofenac has also been associated with the incidence of hepatic injury which is often described as idiosyncratic toxicity [15]. Compared with other NSAIDs, it is well tolerated by the human system and rarely causes serious side effects. Hence, diclofenac has been considered as the drug of 'first choice' in the treatment of chronic and acute inflammatory and painful conditions. In view of clinical importance of diclofenac this drug has been determined by a simple and rapid technique. Many analytical methods for the determination of diclofenac or together with its metabolites in biological fluids and pharmaceutical preparations are reported in literature [16-27]; however, the elucidation of the mechanism of oxidation has attracted little attention. Most of the methods reported for the determination of diclofenac suffer from disadvantages such as laborious, time-consuming and tedious liquid–liquid extraction or solid-phase extraction as sample preparation procedures, long response time, requirement of expensive instruments and low detection capability. Thus, a need was felt for studying the mechanism of oxidation of diclofenac and development of a simple, selective, inexpensive and reliable method for its assay in pharmaceuticals and biological fluids. In the present investigation, square wave voltammetry is used for the routine determination of diclofenac, which has proved to be a versatile technique for the determination of biologically important organic compounds including drugs and related molecules. The developed method is recommended for clinical investigations based on determination of diclofenac levels in medicinal and biological samples. In this section of chaper, an attempt has also been made to characterize the product obtained after oxidation of diclofenac and a tentative mechanism for its formation has been proposed.

Acetyl salicylic acid (ASA) and 1,3,7-trimethylxanthine are important analgesic drugs that alleviate pain without obstructing the conduction of nerve impulse and significantly alter the functions of some sensory organs [28-30]. ASA (II) also known as aspirin inhibits the enzyme cyclooxygenase with consecutive reduction of prostaglandin and thromboxanes [31]. ASA is generally used to treat different types of pain , such as , headache, muscle aches, arthritis, menstrual cramps, backache and sinus infection and is also effective in Alzheimer' disease and cardiovascular diseases [32-35]. 1,3,7-trimethylxanthine, also known as caffeine (CAF, III), is an alkaloid having the basic properties of an organic amine found in food products such as coffee, tea, yerbamate, guarana berries, colanuts and cacao beans [36]. It is the most commonly used stimulant

drug due to its ability to arouse the central nervous system (CNS) and cardiovascular system [37, 38]. CAF is usually prescribed as analgesic adjuvant in pharmaceutical preparations for the treatment of headache and pain related to postpartum, postoperativeand dental surgery [39, 40] and is also therapeutically used for the treatment of migraine in combination with ergotamine [41]. CAF also acts as a natural pesticide as it kills the insects that attempt to feed on plants [42] and is the main and active ingredient of coffee and plays an important role in determining the quality of coffee beverages [43]. Caffeine stimulates the central nervous system by increasing the release of adrenaline and then increases the use of body fat as fuel and spares glycogen [44]. This CNS excitatory response is used by many professional endurance athletes to give them that alertness and sense of extra energy needed for their workouts; hence, caffeine is banned by the World Anti Doping Agency at a level of 12 $\mu g\ mL^{-1}$ in urine [45]. Combination drugs, consisting of aspirin and caffeine, are usually employed for analgesic and antipyretic effects and caffeine is the main and active ingredient of coffee, tea and cola nuts and also used as doping agent; hence, the determination of both the drugs in human body fluids is of great importance. Owing to the common use and misuse and important effects of both analgesics drugs in human system, the development of a fast, sensitive and reliable method for simultaneous monitoring of their trace quantities in human body fluids and pharmaceutical preparations is still highly needed.

Several techniques have been employed for analyzing ASA and CAF individually or simultaneously including spectrophotometric methods, chromatographic methods and solid phase analysis [46-50]. These methods are usually very expensive; more complicated and long time is required for derivatization, extraction and purification of the species prior to their determination using these methods. Electrochemical methods including voltammetric techniques have also been developed for the individual and simultaneous determination of aspirin and caffeine using modified electrodes [32, 33, 36]. These reports need extra time consuming modification process that involves various steps in incorporation of the modifier to the substrate. Moreover, literature survey reveals that very few electroanalytical methods are available for the determination of caffeine since it oxidizes at high positive potential using conventional electrodes; hence, there can be a possibility of overlapping with the potential of discharged electrolytic solution leading to poorly reproducible results [37]. Square wave voltammetry using unmodified EPPGE has been employed in the proposed method for the determination of aspirin and caffeine in drug preparations, human urine samples and coffee beverages. It was observed that oxidation of ASA

and CAF at unmodified EPPGE lead to well-defined peaks and their simultaneous determination was possible.



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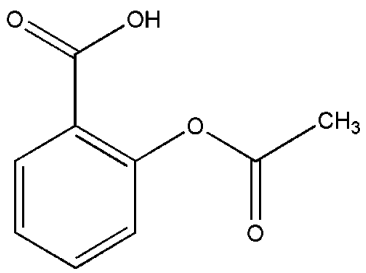

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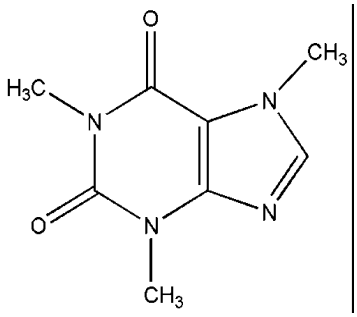


[III]

6.2 EXPERIMENTAL

6.2.1 Instrumentation

The details of the instrumentation used in the studies are essentially similar to the ones reported in earlier chapters. CPE for diclofeanc investigation was performed in a three component cell. A pyrolytic graphite plate (6 cm × 1 cm) was used as working electrode, platinum gauze (cylindrical, diameter ~ 2.5 cm) as auxiliary electrode and Ag/AgCl as reference electrode. The UV-Visible spectral changes were monitored with the help of (Perkin-Elmer-Lambda 35) UV-Vis Spectrophotometer. Lyophilization of the electrolyzed solution was achieved using Lyophilizer (Lab Model) and was purchased from Harrison Scientific Instruments Co. Delhi. The ^{1}H NMR spectrum of the product was recorded in DMSO-d_6 with TMS as an internal standard using (Avance 500 digital) NMR spectrometer from Brucker (500 MHz). The chemical shifts (δ) observed in the studies are reported in parts per million (ppm) of the applied field.

6.2.2 Chemicals and reagents

Diclofenac diethylamine was obtained as a gift from Ish Medicos Private Limited, Dehradun and used as received. The tablets of diclofenac and injections were obtained from the local market. Aspirin was obtained from Sigma Aldrich, USA and caffeine was purchased from Adams Chemical Company, USA. Phosphate buffers of μ = 1 M of different pH were prepared by

the reported method [51]. Aspirin and caffeine containing tablets were procured from the local market of Roorkee. All the reagents and solvents used were of analytical grade.

6.2.3 Measurement procedure

A 1 μM stock solution of diclofenac diethylamine was prepared by dissolving an appropriate amount of the drug in double distilled water and stored in refrigerator. Dilute solutions were obtained by serial dilution of the stock solution. The solutions were deoxygenated by bubbling high-purity nitrogen for 12 – 15 min before recording the cyclic voltammograms. Cyclic voltammograms were recorded in the sweep range 10 – 1000 mV s^{-1} with initial sweep to positive potentials. For square wave voltammetry, optimized parameters used for diclofenac were: initial (*E*): 0 mV, final (*E*): 800 mV, square wave amplitude: 25 mV, potential Step (*E*): 4 mV and square wave frequency: 15 Hz.

Stock solutions of 1 mM of ASA and CAF were prepared by dissolving the desired amount in double distilled water. Required amount of the stock solution was added to electrolytic cell containing 2 mL of phosphate buffer and the total volume was made 4 mL with double distilled water. Optimized parameters of SWV for CAF and ASA were: initial (*E*): 800 mV, final (*E*): 1600 mV, step (*E)*: 4 mV, square wave amplitude: 20 mV, square wave frequency: 15 Hz and sensitivity: 100 μA/V.

6.2.4 Preparation of real samples

The human urine samples of patients undergoing pharmacological treatment with diclofenac were obtained from the Indian Institute of Technology Hospital, Roorkee, after clearance from Ethics Committee of IIT Roorkee. The samples were obtained after 5 h of administration of Voveran tablet containing 100 mg of diclofenac. The anthropometric data of the patients were Sample 1: female, age 30 yrs, height 156 cms, weight 46 kg; Sample 2: male, age 52 yrs, height 170 cms, weight 73 kg and Sample 3: female, age 24 yrs, height 161 cms, weight 55 kg. The samples were used after ten times dilution to reduce the matrix complexity.

Urine sample of heart patients (male: 55 years, 65 kg, 160 cm) and (male: 45 years, 58 kg, 170 cm) undergoing the treatment with ASA (Ecospirin–150) were received from Institute hospital of IIT Roorkee. Samples were then diluted two times with phosphate buffer to reduce the matrix complexity before recording square wave voltammograms. In order to detect caffeine in coffee and tea samples (Instant Coffee of Nescafe classic and Black Tea of Brooke bond Taaza), a known

amount (25 mg) was weighted and dissolved in 25 mL of double distilled water followed by boiling for 1 h at hot plate with stirring. After allowing the residue to settle, the hot solution was filtered and then used for further experiments. A known amount (1 mL) of this solution was added to 1 mL of phosphate buffer of pH 7.2 to record square wave voltammograms.

6.2.5 Oxidation product analysis

The product of the electrooxidation of diclofenac was characterized at pH 7.2. For the identification of oxidation product, about 15 – 20 mg of the compound (I) was exhaustively electrolyzed by applying a potential ~ 100 mV more positive than the oxidation peak potential. The progress of electrolysis was monitored by recording UV spectra and square wave voltammograms at different time intervals. For recording the UV-Vis spectrum, about 2 – 3 mL of the solution from the electrolysis cell was transferred each time to a 1 cm quartz cell and the spectrum was recorded in the range 200 – 800 nm. In spectral studies, two absorbance maxima were observed at λ_{max} 220 nm and 275 nm just before oxidation. With the progress of electrolysis the colourless solution of diclofenac changed to yellow within two hours. An absorbance band at λ_{max} 450 nm was observed thereby indicating that an intermediate is generated which is more extensively π-conjugated than the starting species. The end of electrolysis was indicated by the disappearance of the oxidation peak in square wave voltammogram. The yellow colour slowly disappeared and in the end of electrolysis the solution turned to colourless and the absorbance band at λ_{max} 450 nm disappeared. The exhaustively electrolyzed solution was removed from the cell, filtered using Whatman filter paper 42, lyophilized and extracted using methanol. The methanolic extract exhibited a single spot in TLC indicating the formation of single product. The dried material was analyzed by ^{1}H NMR.

6.3 RESULTS AND DISCUSSION

[1] Voltammetric Determination of Diclofenac

6.3.1 Cyclic voltammetry

Cyclic voltammetry is the most widely used technique as it provides considerable information about the thermodynamics of redox processes, the kinetics of heterogeneous electron-transfer reactions, coupled chemical reactions and adsorption processes; hence, initial studies were carried out using this technique. Cyclic voltammograms were recorded for 500 nM diclofenac at EPPGE at pH 7.2 using a sweep rate of 20 mV s^{-1}. Diclofenac is irreversibly oxidized giving rise to an oxidation peak at ~ 662 mV (I_a) when the sweep was initiated in the positive direction. In the

reverse sweep a peak II_c was noticed which formed a reversible couple with peak II_a observed in the subsequent sweep towards positive potentials. A typical cyclic voltammogram of diclofenac is shown in **Fig. 6.1**. The reversible couple is formed at less positive potentials due to the oxidation product of diclofenac which is electrochemically active.

To ascertain the nature of the reaction, sweep rate studies were performed in the range 10 – 1000 mV s^{-1}. The peak separation between the reversible couple was found to be ~ 20 mV which remained constant with increase in the sweep rate. The ratio of anodic (II_a) and cathodic (II_c) peak currents was found to be equal to unity which remained independent of sweep rate studies. It is inferred from the above results that the reaction is reversible in nature. The analyte peak current was found to increase with increasing sweep rates and the plot of $i_p/v^{1/2}$ versus log v clearly indicated that the electrode process is adsorption controlled [52, 53].

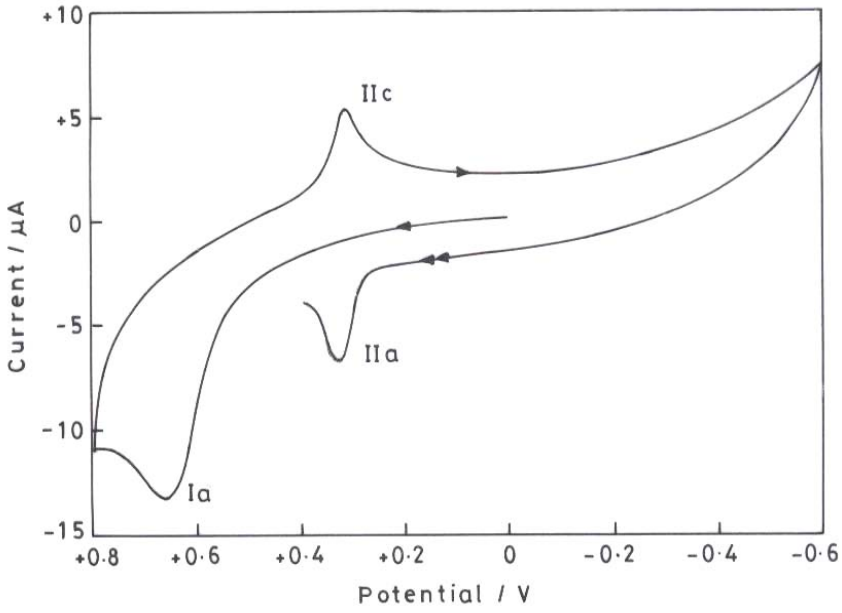


Figure 6.1: Cyclic voltammogram obtained for 500 nM diclofenac at pH 7.2 using EPPGE at 20 mV s^{-1}.

6.3.2 Square wave voltammetry

For analytical purpose, square wave voltammetry is generally the first choice as it suppresses the background current effectively and has higher sensitivity. Initially, square wave voltammograms were recorded for 500 nM diclofenac at basal plane and edge plane pyrolytic

graphite electrode in phosphate buffer of pH 7.2. The drug was oxidized at ~ 674 mV with a very low current value at basal plane electrode. EPPGE was then used as the working electrode at which an oxidation peak was observed at ~ 500 mV with a marked increase in the current. Thus, the exposed edge plane sites in EPPGE contribute in making it an efficient sensor, which enhances the kinetics of the electrochemical process. Hence, further investigations were carried out at EPPGE using SWV.

6.3.2.1 Influence of pH and square wave frequency

The pH of the supporting electrolyte affects the oxidation peak potential of diclofenac. The voltammetric oxidation of 500 nM diclofenac was examined in the range 2.4 – 11.0 in phosphate buffer. The peak potential of the drug shifted towards less positive potentials with increase in pH.

The linear dependence of the peak potential on pH at the EPPGE is represented by the following equation:

E_p (pH 2.4 – 11.0) = [– 59.72 pH + 922] ($R^2 = 0.9986$)

The dE_p/dpH value of ~ 60 mV pH^{-1} indicates that equal number of protons and electrons are involved in the oxidation of diclofenac.

The dependence of peak current and peak potential of diclofenac on the square wave frequency (*f*) was studied in the range 5 – 200 Hz. The peak current was found to increase linearly with square wave frequency and the linear relation between i_p and f can be expressed by the equation:

i_p (10^{-5} A) = 0.1404 f + 1.427 ($R^2 = 0.9938$)

The peak potential of diclofenac shifted towards more positive potential with increase in square wave frequency. The plot of E_p versus log f was linear and the variation can be expressed by the relation:

E_p (mV) = 93.99 log f + 385.58 ($R^2 = 0.9954$)

These observations are in agreement with the properties of irreversible electrochemical process which is adsorption controlled [54-56]. The results supported the inferences obtained from cyclic voltammetry studies.

6.3.2.2 Calibration plot

The effect of concentration of diclofenac on peak current was studied at optimized parameters in the concentration range 10 – 1000 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. 6.2** depicts the systematic increase in the peak current values of the oxidation peak with an increase in the concentration and the linear calibration plot is depicted as the inset of Fig. 6.2. The error bars for five determinations are also included in the inset.

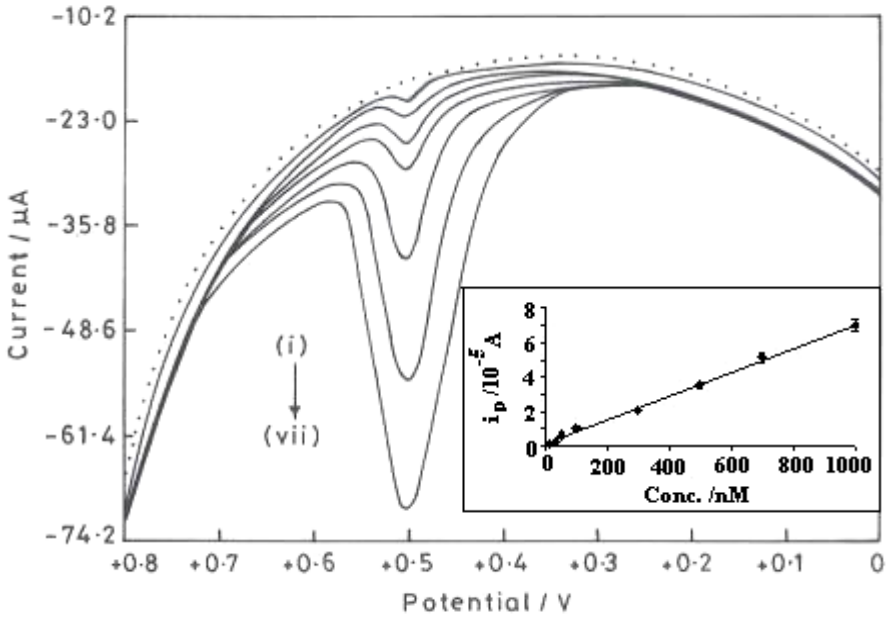


Figure 6.2: Square wave voltammograms recorded for (a) phosphate buffer (background) at EPPGE (·····) and (b) increasing concentration of diclofenac at the electrode (—) [Curves were recorded at (i) 10; (ii) 30; (iii) 50; (iv) 100; (v) 300; (vi) 500 and (vii) 700 nM concentration in phosphate buffer of pH 7.2], inset is showing the calibration plot.

The linearity of peak current versus concentration can be expressed by the following regression equation:

i_p (10^{-5} A) = 0.0069 C (nM) + 0.1705 (R^2 = 0.9977)

where C is the concentration of diclofenac. The sensitivity of the proposed method is 69 nA nM^{-1}. The detection limit was calculated by using the formula $3\sigma/b$, where σ is the standard deviation of the blank and b is the slope of the calibration curve and it was found as 6.2×10^{-9} M.

6.3.3 Interference effect

Selectivity is an important characteristic of an electrode which determines whether a target species concentration can be estimated accurately by using the proposed electrode. In real samples there are many concomitant substances which may affect the selectivity of the proposed method. In order to get better analysis results, the interferences by these compounds were tested. Under the optimized experimental conditions, the effects of uric acid, xanthine, hypoxanthine and ascorbic acid on oxidation of 100 nM diclofenac were evaluated. The tolerance limit was defined as the concentrations of foreign substances, which gave an error less than ± 5 % in the detection of diclofenac. The results showed that 10-fold concentration of the above compounds did not interfere with the determination of the drug. This indicates that the method can be safely applied to the determination of diclofenac in biological fluids.

6.3.4 Pharmaceutical analysis

In order to demonstrate the applicability of the proposed method to the determination of diclofenac, the method was applied to the analysis of the drug in various samples of pharmaceutical preparations. The medicinal samples containing diclofenac were Voveran (Ankur Drugs and Pharma Ltd., Solan, Himachal Pradesh), NAC SR-100 (ONTOP Pharmaceuticals Ltd., Bangalore), Dynapar (Treikas Pharmaceuticals Ltd., Dehradun, Uttaranchal) and Panjim (Embark Lifescience Pvt. Ltd., Roorkee). The tablets were powdered and homogenized. Solutions obtained by dissolution of tablets and injections were diluted so that diclofenac concentration lies in the range of calibration curve. Square wave voltammograms were then recorded at EPPGE and keeping the dilution factor in consideration, the concentration of diclofenac in the pharmaceutical formulations was determined. In **Table 6.1**, results obtained for the concentration of diclofenac are summarized. The results were found in satisfactory agreement with the labelled amounts.

6.3.5 Real sample assay

To establish the utility of the developed protocol, diclofenac was determined in human urine samples obtained after the usual therapeutic dose of diclofenac has been administered. The

samples were obtained from patients after 5 h of administration of Voveran tablet containing 100 mg of diclofenac. Prior to analysis, the urine samples were diluted ten times with phosphate buffer. A typical square wave voltammogram of sample 1 at EPPGE is shown in **Fig. 6.3.** A well-defined peak of diclofenac was noticed at E_p ~ 500 mV. The other voltammetric peak at ~ 300 mV is estimated to be due to the presence of uric acid in the urine sample. The urine sample of the patient was then spiked with a known concentration of diclofenac. The voltammogram in Fig. 6.3 clearly depicts that the peak current increases significantly for the peak at E_p ~ 500 mV thereby confirming that it corresponds to the oxidation of diclofenac. The concentration of diclofenac was determined using the regression equation. Using the proposed method described above, the results obtained for different urine samples, before and after spiking, are tabulated in **Table 6.2**.

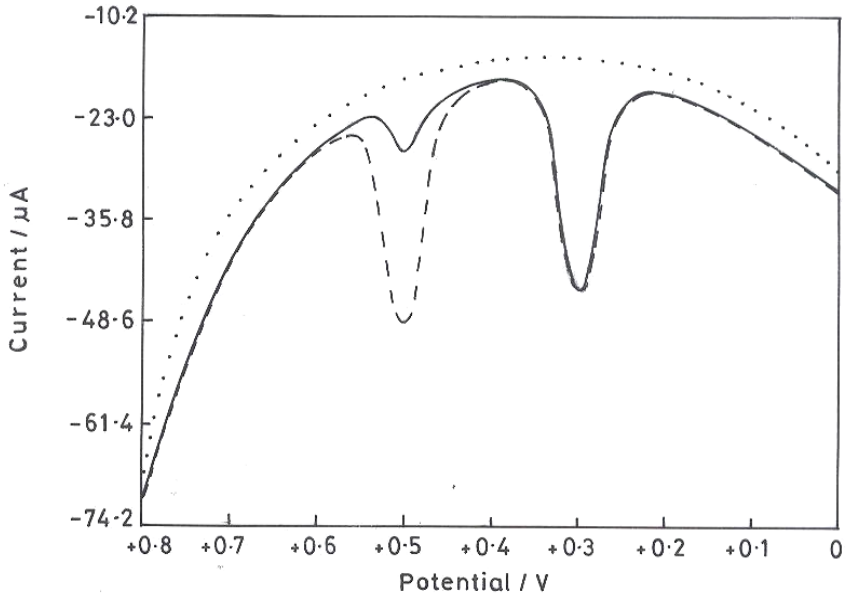


Figure 6.3: A comparison of voltammograms observed for urine sample of patient being treated with diclofenac (—) and the patient sample spiked with diclofenac (- - - -) at pH 7.2 at EPPGE. Background is represented as (….).

6.3.6 Product characterization

The exhaustively electrolyzed product of diclofenac oxidation was characterized using ^{1}H NMR. The results obtained from UV spectra clearly indicated that during the oxidation of diclofenac an intermediate is generated which shows λ_{max} at 450 nm. The other two bands at λ_{max} 220 nm and 275 nm of diclofenac may be attributed to the π-π^* transition and benzenoid band, respectively. The ^{1}H NMR of the material in DMSO exhibited signals at δ_H (500 MHz; DMSO-d_6;

Me_4Si) 6.34 (1H, d), 6.51 (1H, dd), 6.72 (1H, d), 7.01 (1H, t) and 7.28 (2H, d). The methylene protons exhibited a singlet at 3.74. The ^1H NMR data suggested the formation of 5-OH diclofenac as the oxidized product.

Table 6.1: Determination of diclofenac in pharmaceutical preparations using EPPGE.

Sample	Stated content	Determined content	Error (%)
Voveran	100 mg	98.95 mg	- 1.05
NAC SR-100	100 mg	97.04 mg	- 2.96
Dynapar	75 mg/mL	73.62 mg/mL	- 1.84
Panjim	25 mg/mL	24.16 mg/mL	- 3.36

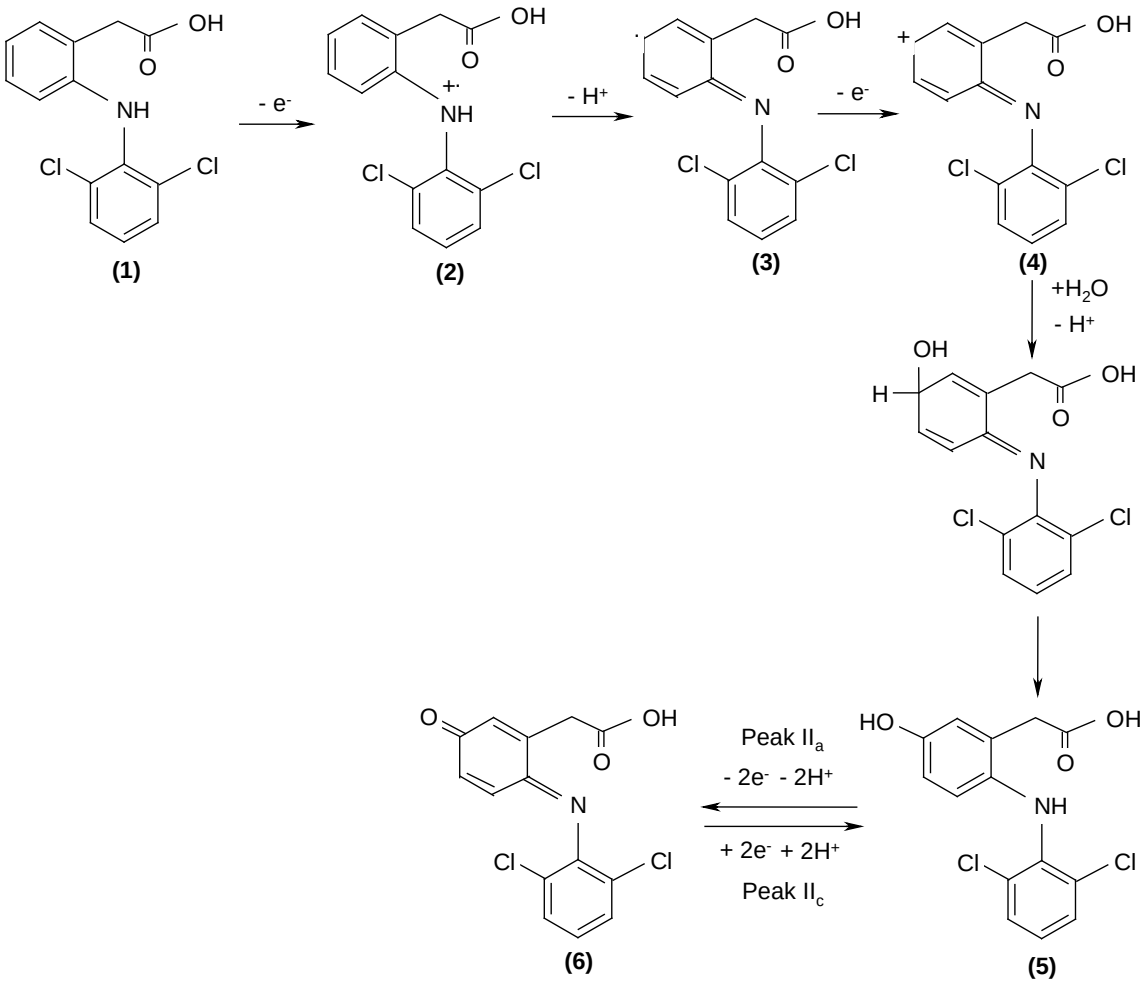
Table 6.2: Concentration of diclofenac in human urine after 5 h of diclofenac administration at edge plane pyrolytic graphite electrode.

	Spiked (µM)	Detected (µM)	Recovery (%)
Sample 1	0.00	1.22	—
	1.00	2.25	101.35
	3.00	4.31	102.13
	5.00	6.18	99.36
Sample 2	0.00	1.21	—
	1.00	2.22	100.45
	3.00	4.07	96.67
	5.00	6.28	101.13
Sample 3	0.00	1.23	—
	1.00	2.19	98.21
	3.00	4.12	97.40
	5.00	6.31	101.28

The number of electrons (n) involved in the electrooxidation of diclofenac was determined by monitoring the exponential decay of the current-time curve. The exhaustive electrolysis normally required 8 to 10 h and the electrode surface had to be cleaned number of times to achieve

faster electrolysis. The plot of i_p vs. time was exponential and average experimental value of number of electrons involved were 2.0 ± 0.2 per mole at pH 7.2.

The tentative mechanism for the electrochemical hydroxylation of diclofenac is shown in **Scheme 1**. The initial step appears to involve an electron abstraction, which leads to a nitrogen centered radical cation (2). The radical cation undergoes rearrangement and deprotonation leading to a carbon centered radical para to the amino group (3). The radical then undergoes a second electron abstraction leading to a carbocation (4), which reacts with water and by aromatization of the intermediate, 5-OH diclofenac (5) is formed. The yellow colour of the electrolyzed solution after two hours of the start of reaction is assigned to the formation of cation radical which exhibits an absorption band at λ_{max} 450 nm. The formation of cation free radical in the copolymer prepared from 3, 4-ethylenedioxythiophene and diclofenac has also been reported to exhibit yellow colour with absorption maxima at 474 nm [57]. Thus, diclofenac is oxidized to 5-OH diclofenac by a loss of 2e, $2H^+$ process. 5-OH diclofenac is able to form quinone imine (6) by a reversible process of 2e, $2H^+$. This reaction is supported by the data obtained from the cyclic voltammetry. The reversible couple as can be seen in Fig. 6.2 is due to the formation of diclofenac-2, 5-quinone imine.



Scheme 1: A tentative mechanistic pathway proposed for the electrooxidation of diclofenac at pH 7.2 at EPPGE.

[2] Voltammetric Determination of Aspirin and Caffeine

6.3.7 Cyclic voltammetry

Electrochemical response of a solution having 5 µM of each ASA and CAF was estimated by cyclic voltammerty at 50 mV s^{-1} under optimized parameters using EPPGE. Anodic peaks obtained for the oxidation of ASA and CAF were observed at ~ 1272 and ~ 1410 mV, respectively as shown in **Fig. 6.4**. The absence of any reduction peak in the reverse sweep for both the compounds clearly indicates the irreversibility of the electrode reaction. Square wave voltammetry is more sensitive technique with well established advantages such as discrimination against background current, low detection limit and high sensitivity, hence; further study for the analysis of aspirin and caffeine in real samples was carried out by using this technique.

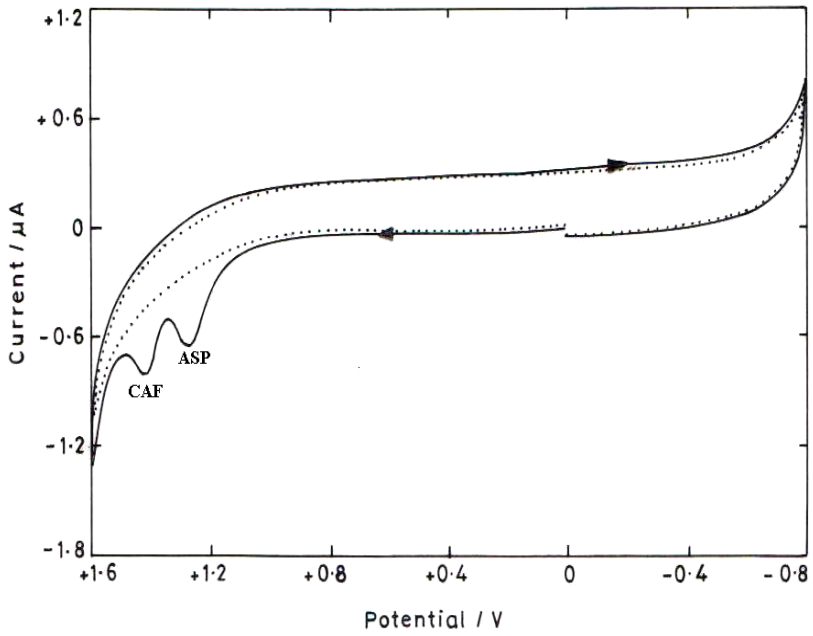


Figure 6.4: Cyclic voltammograms of homogeneous solution of ASP and CAF in phosphate buffer of pH 7.2 using edge plane pyrolytic graphite electrode (—) at scan rate of 50 mV s^{-1} and dotted CV (…..) is the response of EPPGE in blank solution.

6.3.8 Square wave voltammetry

6.3.8.1 Effect of pH

The pH of supporting electrolyte is an important factor that affects redox behavior of biomolecules and drugs. The effect of pH on oxidation peak potential of ASA and CAF was evaluated in the pH range of 4.3 – 10.9. It was observed that peak potential of both the compounds

shifted to less positive potential with increase in pH as shown in **Fig. 6.5 a**. The E_p vs. pH plots are linear and dependence of anodic peak potential of both analytes on the pH of supporting electrolyte can be presented by the following equations:

E_p /mV [4.3 – 10.9] = - 29.94 pH + 1418 versus Ag /AgCl for ASA

E_p /mV [4.3 – 10.9] = - 49.35 pH + 1666 versus Ag /AgCl for CAF

having correlation coefficients 0.983 and 0.989, respectively. The value of dE_p /dpH ~ 30 mV/pH for ASA indicates that number of protons involved in oxidation are one half of the electrons ($2e^-$, $1H^+$), whereas, dE_p/dpH value of 49.35 mV for CAF indicates the involvement of equal number of protons and electrons ($4e^-$, $4H^+$) in the oxidation reaction of CAF [32, 58].

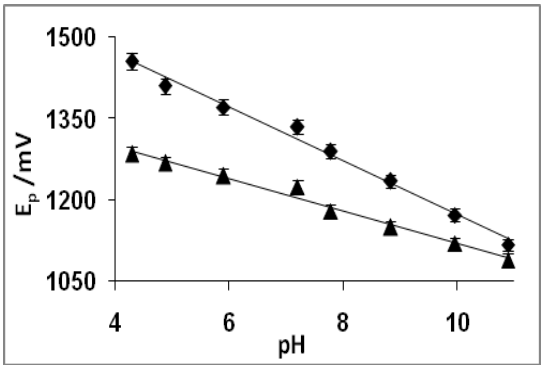
6.3.8.2 Effect of square wave frequency

The variation of square wave frequency with peak current of ASA and CAF was studied in the frequency range of 5 – 50 Hz at pH 7.2. The peak current of 15 µM L^{-1} aspirin and caffeine shows a linear increase with square root of square wave frequency as shown in **Fig. 6.5 b and c**, respectively, suggesting thereby that electrode reaction for both the analytes is diffusion controlled process [59]. Linear relations between i_p and $f^{1/2}$ for both the drugs can be expressed by the following equations:

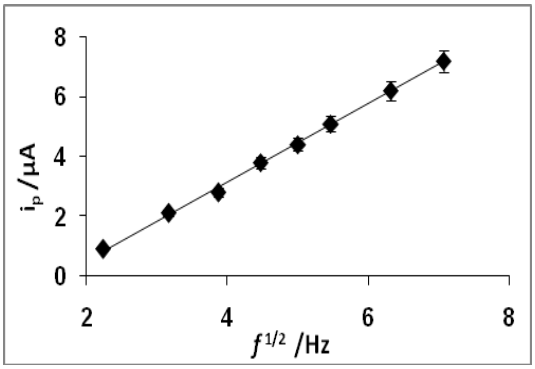
i_p /µA = 1.308 $f^{1/2}$ (Hz) - 2.087 for ASA

i_p /µA = 1.940 $f^{1/2}$ (Hz) - 4.136 for CAF

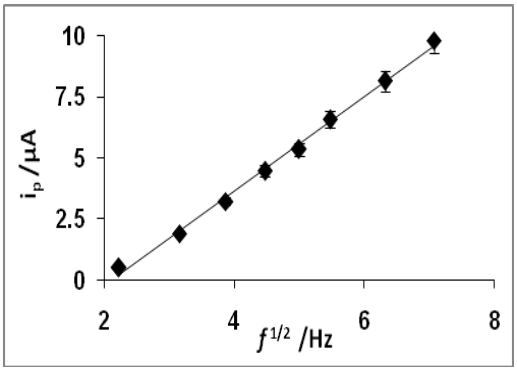
with correlation coefficient of 0.998 and 0.996 for ASA and CAF, respectively.



(a)



(b)



(c)

Figure 6.5: (a) Observed dependence of peak potential on pH for ASA (▲) and CAF (■), (b) Plot of i_p versus square wave frequency for aspirin, (c) plot of i_p versus square wave frequency for caffeine using EPPGE.

6.3.8.3 Effect of concentration

It is well known that peak current depends on the concentration of analytes; hence, square wave voltammograms were recorded for various concentrations of ASA and CAF in phosphate buffer of pH 7.2. It was observed that with increasing concentration of drugs, oxidation peak current was found to increase linearly. The oxidation peak current versus concentration plots showed a good linearity for ASP and CAF in the concentration range of 0.02 – 100 µmol L^{-1} as depicted in insets of Fig. 6.6 and Fig. 6.7, respectively. Linear regression equations for both drugs arising from calibration plots can be represented as:

i_p (µA) = 0.163 C (µmol L^{-1}) + 0.043 versus Ag/AgCl for ASA

i_p (µA) = 0.174 C (µmol L^{-1}) + 0.086 versus Ag/AgCl for CAF

with a correlation coefficients of 0.993 and 0.997, respectively. The limits of detection were found to be 0.1 × 10^{-7} and 0.08 × 10^{-7} M for ASA and CAF, respectively. The detection sensitivities for ASA and CAF were found to be 0.16 and 0.17 µA/µmolL^{-1}, respectively and the limits of quantification were calculated as 0.32 × 10^{-7} and 0.26 × 10^{-7} M, respectively. Since, for any analytical method it is advantageous to calculate validation parameters hence, the important calibration characteristics are determined and are given in **Table 6.3**.

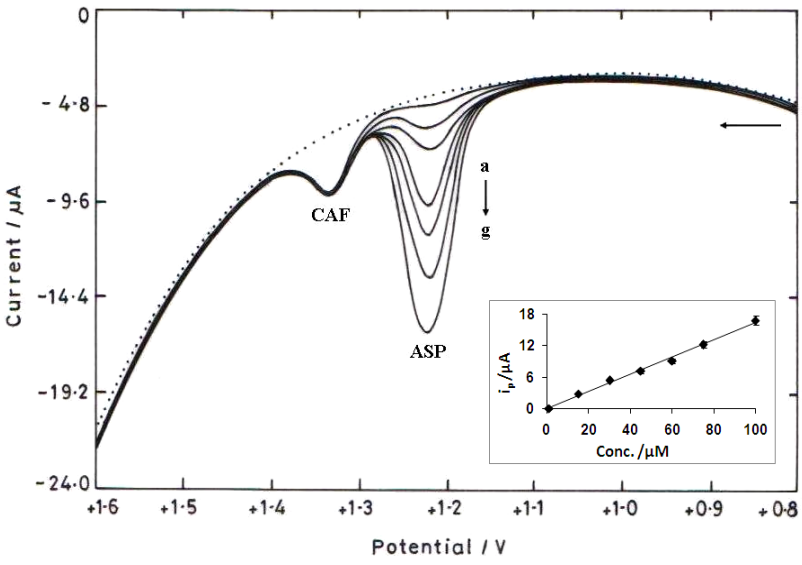


Figure 6.6: **Square wave voltammograms observed for phosphate buffer (background) at EPPGE (…..) and increasing concentration of ASP at a fixed concentration of CAF; [CAF] = 15 µM; [ASP]: a=1, b=10, c=15, d=30, e=45, (f) 60 and (g) 75µM. Inset is calibration curve for aspirin.**

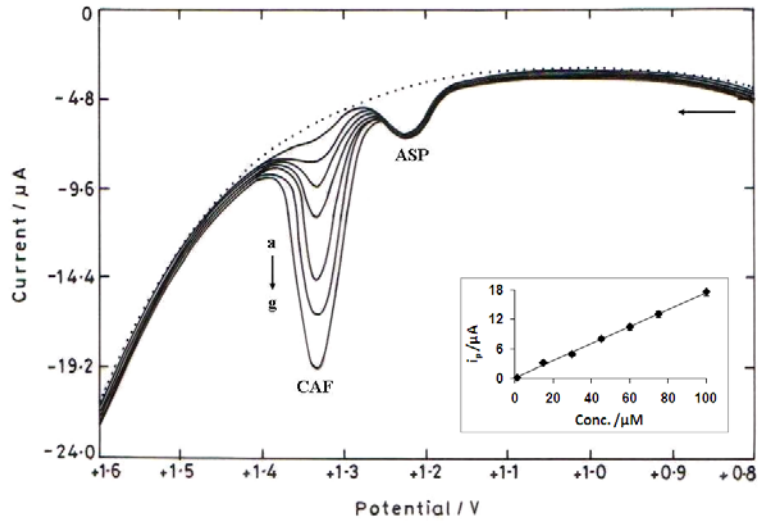


Figure 6.7: **Square wave voltammograms observed for phosphate buffer (background) at EPPGE (…..) and various concentrations of CAF at a constant concentration of ASP; [ASP] = 15 µM; [CAF]: a=1, b=10, c=15, d=30, e=45, (f) 60 and (g) 75µM. Inset is calibration curve for caffeine.**

6.3.9 Simultaneous determination of ASA and CAF

Square wave voltammograms are recorded to evaluate the electrochemical response of different concentration of ASA and CAF, when both the substances are present in the same solution. For this purpose firstly, the concentration of CAF was kept constant at 15 µmol L^{-1}, while concentration of ASP was varied from 1 to 75 µmol L^{-1} as shown in **Fig. 6.6**. Analogously, as shown in **Fig. 6.7**, the influence of CAF concentration was checked by increasing the CAF concentration from 1 to 75 µmol L^{-1} while fixing the ASP concentration constant at 15 µmol L^{-1}. Examination of the obtained results indicates that the oxidation peak current for ASA systematically increases with increasing concentration at a constant concentration of CAF (whose oxidation peak current is remained fairly constant). Similarly oxidation peak current for CAF systematically increases as its concentration increased at a constant concentration of ASA (whose oxidation peak current is remained constant). When CAF and ASA were present in the same solution, SWVs revealed that when concentrations of both compounds are increased simultaneously, both compounds exhibit oxidation peaks separately without interfering each other. It was also found that oxidation peaks observed for ASA and CAF in same solution do not interfere with each other using EPPGE and also the peak current values are exactly similar to those received from individual calibration plots of ASA and CAF. Thus, it can be concluded that the proposed sensor can be successfully applied for the simultaneous determination of ASA and CAF in real samples. However, because of unavailability of such type of samples, ASP and CAF were analyzed individually in real samples using proposed sensor.

Table 6.3: The calibration characteristics for aspirin and caffeine using edge plane pyrolytic graphite electrode.

Analytical Parameter	Aspirin	Caffeine
Calibration range (µM)	0.02–100	0.02–100
Correlation coefficient (r^2)	0.993	0.997
Measured Potential (mV)	1225	1335
Detection limit (µM)	0.01	0.008
Limit of quantification (µM)	0.032	0.026
Sensitivity (µA/µM)	0.16	0.17
RSD of slope %	1.0	2.9
RSD of intercept %	0.8	1.2
Repeability of peak current (RSD %)	0.32	0.54
Repeability of peak potential (RSD %)	0.62	0.68
Reproducibility of peak current (RSD %)	0.70	0.89
Reproducibility of peak potential (RSD %)	1.40	1.48

6.3.10 Analytical applicability

6.3.10.1 Analysis of pharmaceutical preparations

The determination of ASA and CAF contents in commercial tablets was carried out using EPPGE. Firstly the tablets were weighed accurately, grounded into powder and then dissolved into double distilled water. An adequate amount of this solution was diluted suitably with phosphate buffer so that it comes into the linear concentration range. SWVs were then recorded under optimized parameters and the concentration of aspirin and caffeine content in tablets was determined using proposed method. The experimentally detected values and the labeled values are compared in **Table 6.4** and it is found that the results obtained using proposed sensor are in concordance with the claimed amount within the error of ± 4.3 % . Therefore, it is recommended that proposed sensor is very useful and can be employed successfully for the determination of aspirin and caffeine in pharmaceutical preparations.

Table 6.4: Determination of ASA and CAF in pharmaceutical tablets using EPPGE.

Compound	Tablet name / company	Reported amount (mg)	Detected amount (mg)	Error%
Aspirin	**Ecospirin-150** (USV Ltd., Govandi, Mumbai)	150	152.99	+ 1.99
	Disprin (Reckitt Benckiser Ltd. Hootagalli, Mysore)	350	334.83	– 4.33
Caffeine	**Anacin** (Mfd. by Wyeth Ltd., India)	30	31.19	+ 3.96
	Sinarest (Centaur Pharm. Pvt. Ltd., Mapusa, Goa)	30	28.95	– 3.50

6.3.10.2 Analysis of human urine samples

Aspirin is clinically employed for analgesic and antipyretic effects and some amount of unmetabolized drug usually excretes in patient urine [33]. Hence, it is considered worthwhile to find out the concentration of aspirin in urine sample of heart patients undergoing treatment with aspirin. For this study, urine samples of patients were diluted two times with phosphate buffer before recording square wave voltammograms in order to reduce the complexity arising from matrix. A small anodic peak (E_p ~ 1225 mV) was noticed for the oxidation of ASA in patient's urine sample along with some other peaks as shown in **Fig. 6.8**, indicating thereby the presence of aspirin in urine sample of patient prescribed with Ecospirin-150. No attempts have been made to identify the other peaks, which may be due to the oxidation of common urinary compounds such as xanthine, uric acid, ascorbic acid and other drugs taken by patient with Ecospirin-150. Further, standard addition method was employed to reconfirm the actual concentration of drug in two urine samples. For this purpose spiking was carried out in diluted urine samples with known concentrations of ASA followed by recording SWVs under identical conditions. The actual

concentration of ASA in urine sample of patient undergoing treatment with aspirin was evaluated by using calibration curve and observed to be 0.3×10^{-7} M with relative standard deviation of ± 3.6%.

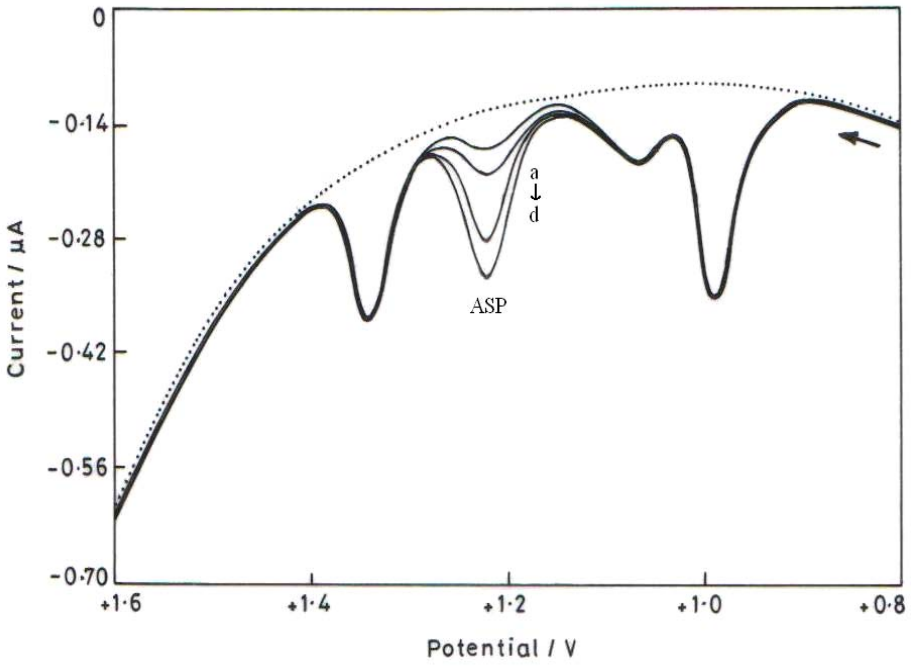


Figure 6.8: Square wave voltammograms observed for (a) urine sample of patient taking prescription of Ecospirin-150; (b to d) urine sample after spiking with 0.2, 0.7 and 1.0 µM of standard aspirin respectively, at pH 7.2 using EPPGE.

6.3.10.3 *Analysis of caffeine containing beverages*

Caffeine is the main and active ingredient of coffee, tea and cola nuts; hence, it was considered that determination of its concentration in caffeine containing food samples for quality control purposes would be advantageous. A known amount (1 mL) of caffeine solution prepared according to the procedure mentioned in experimental section was added to 1 mL of pH 7.2 phosphate buffer to record square wave voltammograms. The samples were diluted suitably so that the concentration fall in the range of calibration curve and then square wave voltammograms were recorded under optimum conditions and parameters. The square wave voltammogram of coffee sample is presented in **Fig. 6.9**. Average value of three repetitive measurements of oxidation peak current was used to determine the actual concentration of caffeine in coffee and tea samples with

the help of regression equation. Concentration of caffeine in coffee and tea samples was found to be 0.8 µM mg^{-1} mL^{-1} and 0.6 µM mg^{-1} mL^{-1}, respectively.

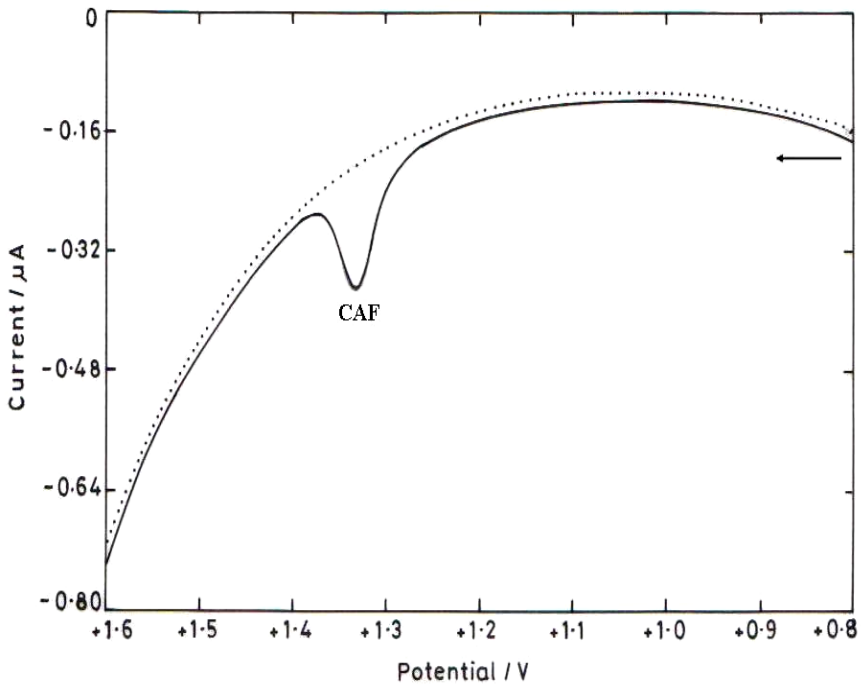


Figure 6.9: Square wave voltammograms for coffee sample at pH 7.2 using EPPGE.

Table 6.5: Comparison between the proposed sensor and earlier reported sensors for the voltammetric determination of ASA and CAF.

Sensor used	Concentration range (µM)	Detection limit (µM)		Reference
		ASA	CAF	
NaMM/GCE	0.22 – 1.66	0.10	–	28
Surfactant-MWCNT/CPE	0.29 – 60.00	0.08	0.090	32
MWCNT-ACS/GCE	15.00 – 65.00	3.77	–	33
MIP/CPE	0.06 –250.00	–	0.010	36
CME	5.00 – 200.00	–	2.000	39
BDD	9.70 –110.00	–	7.000	41
BQMCPE	0.00 –500.00	–	0.300	42
Nafion–MWCNT/GCE	0.60 – 400.00	–	0.200	58
Bare EPPGE	0.02 –100.00	0.01	0.008	Proposed method

6.4 CONCLUSIONS

The proposed protocol demonstrates the successful application of edge plane pyrolytic graphite electrode for the determination of diclofenac, ASA and CAF in pharmaceuticals products, human urine samples and coffee and tea beverages with excellent sensitivity and selectivity. The electrochemical oxidation of diclofenac occurs in 2e, $2H^+$, pH dependent peak. The product of oxidation has been characterized as 5-hydroxydiclofenac using ^1H NMR. The quantitative determination of diclofenac was carried out in the range 10 – 1000 nM with a low detection limit of 6.2×10^{-9} M. The analysis of medicinal samples of diclofenac using the proposed method was satisfactory as the detected content was in good agreement with the reported values. The practical utility of the present method was successfully examined by analyzing urine samples obtained from patients being treated with diclofenac.

In case of aspirin and caffeine, the most of the recently reported sensors **(Table 6.5)** have comparatively high detection limit, which is of less importance in trace analysis cases. Moreover,

these reported methods require complicated surface modification procedure with different kinds of surface modifiers, while, in proposed method EPPGE was used without any surface modification or pretreatment. It is well known that the exposed sites of the edge plane pyrolytic graphite electrode contribute to its efficiency leading to the lowering of oxidation peak potential and marked enhancement in the peak current [60]. Thus, such an electrode considerably improves current response (sensitivity) of both analyte as compared to other reported methods (Table 3). The proposed sensor has also been utilized successfully for the analysis of urine samples of patients, prescribed with aspirin and caffeine to determine unmetabolized drugs present in urine samples with no special pretreatment of samples except suitable dilution. Hence, proposed sensor can be strongly recommended for detecting doping cases at the site of competitive games due to its fastness and accuracy. The proposed method raises the possibility for exploring these drugs separately or in combination with other drugs that follow same pathways with the hope of finding better ways to control pain and doping cases. The proposed sensor is also likely to be useful device for quality control analysis in food chemistry and pharmaceutical industries.

It is thus concluded from the above investigations that the proposed method is beneficial for the sensitive determination of diclofenac, aspirin and caffeine owing to its simplicity, specificity, selectivity and relatively short analysis time.

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