

Development of Rapid Testing Technique to Diagnose Antibiotic Resistance



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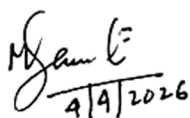
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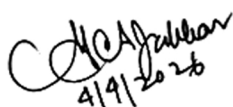
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Declaration

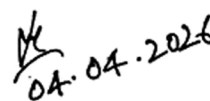
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Dedicated
to
the victims of this earth suffered severely from anti-microbial
resistance

Abstract

Antibiotic resistance is a rapidly growing global health concern, creating an urgent need for detection methods that are both rapid and reliable. Antibiotic resistance occurs when microorganisms survive or grow despite exposure to antibiotic drugs designed to kill or inhibit them. It happens due to several factors like inappropriate prescribing, overuse, misuse, antibiotic residue intake from food items like eggs, meats, vegetables. Recent data shows, approximately 1.27 million deaths occurring each year, and the figure could increase to 39 million by 2050 if failed to take action. Besides, the overuse and misuse of antibiotics have led to the rise of multidrug-resistant organisms, commonly known as "superbugs," which are resistant to multiple antibiotics and complicate the treatment. The economic impact is also alarming. World Bank is projecting potential global GDP losses of up to \$3.4 trillion annually by 2030 due to the antibiotic resistance.

Traditional methods for detecting antibiotic resistance primarily involve phenotypic tests like disk diffusion, broth/ agar dilution, e-test and some automated systems that observe the growth response of bacteria to antibiotics. Though traditional methods are widely used for detecting antibiotic resistance but it has several drawbacks like time-consuming, labor-intensive, variability in data or results. The main challenge for the doctors is getting test data prior to prescribe right antibiotics to the patient. Considering the patient situation, most of the cases doctor prescribe antibiotic based on the assumption.

This doctoral research introduces an electrochemical sensing strategy for the detection of antibiotics and the assessment of resistance phenomena, built upon a poly-*L*-glutamic acid (PGA) modified glassy carbon electrode (GCE). The objective is to seek a rapid and reliable detection mechanism of antibiotic resistance.

Aligning with the research objectives, the work is divided into four experimental phases, each addressing a distinct objective in sensor development and application like antibiotic determination and evaluation of resistance phenomena.

In the first phase, the glassy carbon electrode surface was modified through electropolymerization of *L*-glutamic acid to form a PGA coating. The modification was confirmed through a combination of analytical techniques. Cyclic Voltammetry (CV) demonstrated enhanced redox activity of the PGA modified GCE as compared to the bare GCE. Infrared (IR) spectroscopy revealed characteristic peak shifts consistent with polymer formation. Electrochemical Impedance Spectroscopy (EIS) showed reduced charge transfer resistance and improved sensitivity in Nyquist plots. Scanning Electron Microscopy (SEM) visualized a uniform polymer layer with increased surface roughness. These

results verified the successful fabrication of PGA modified electrode with a highly responsive electrode surface.

In the second phase of the study, we targeted to apply the PGA modified electrode to the detection of Ceftributen, a β -lactam antibiotic. Versatile CV and Differential Pulse Voltammetry (DPV) were employed in phosphate buffer (pH 6.8), enabling trace-level detection of Ceftributen. A model experiment for the detection of Ceftributen was carried out; ie., in-vitro experiments with blood serum confirmed the method's applicability in complex biological matrices. A simulation study incorporating β -lactamase enzyme demonstrated the sensor's ability to detect antibiotic degradation, as indicated by a marked reduction in peak current corresponding to β -lactam ring cleavage. This will ultimately, facilitate to recognize the extend clinical study to determine the antibiotic content in blood or urine samples thus the detection of antibiotic resistance.

The third phase was extended for the approach applying the same system to Cefuroxime, another β -lactam antibiotic, following the same methodological framework. The PGA-modified GCE again exhibited high sensitivity in both buffer and serum samples. Enzymatic degradation by β -lactamase produced a consistent decline in electrochemical signal, confirming the PGA modified GCE capability to monitor resistance-related biochemical changes.

In the fourth phase, the PGA modified GCE was evaluated for Levofloxacin, a fluoroquinolone antibiotic. Trace detection was of Levofloxacin was achieved in buffer and serum as well as in a simulation study using *Escherichia coli* ATCC culture sample containing Levofloxacin revealed a significant drop in peak current after bacterial exposure, indicating detection of antibiotic interaction and potential resistance development for the antibiotics. This demonstrated a platform for the versatility for the detection of the resistance of both β -lactam and non- β -lactam antibiotics.

Overall, the findings establish PGA-modified GCE-based electrochemical system as rapid, sensitive, and dependable tools for antibiotic detection and their resistance assessment. Unlike conventional microbiological assays, which often require more than 24 hours, this approach can deliver actionable results within 2-4 hours, offering a substantial advantage for timely clinical decision-making. The outcomes of this research provide a foundation for future development of point-of-care diagnostic systems and encourage further investigation across a wider range of antibiotic classes and resistance mechanisms.

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List of Abbreviations

BC	Before Christ
CE	Common Era.
TCM	Traditional Chinese Medicine
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
AMR	Antimicrobial Resistance
WHO	World Health Organization
HIV	Human Immunodeficiency Virus
AIDS	Acquired Immunodeficiency Syndrome
LMICs	Low- and Middle-Income Countries
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
PCR	Polymerase Chain Reaction
qPCR	Quantitative PCR (Polymerase Chain Reaction)
NGS	Next-Generation Sequencing
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight mass
MS	spectrometry
MBC	Minimum Bactericidal Concentration
In Vitro	Latin for “in glass,” referring to experiments performed in a controlled environment outside a living organism
In Vivo	Latin for “within the living,” referring to experiments conducted inside a living organism
<i>E. coli</i>	<i>Escherichia coli</i>
ATCC	American Type Culture Collection
PBPs	Penicillin-binding Proteins
CV	Cyclic Voltammetry
DPV	Differential Pulse Voltammetry
LSV	Linear Sweep Voltammetry
EIS	Electrochemical Impedance Spectroscopy
SWV	Square Wave Voltammetry
FT-IR	Fourier Transform Infrared
SEM	Scanning Electron Microscope
SE	Secondary electrons
BSE	Backscattered

GCE	Glassy Carbon Electrode
PGA	Poly-glutamic Acid
LC-MS	Liquid chromatograph-mass spectrometry
MOFs	Metal-organic frameworks
MIPs	Molecularly imprinted polymers
API	Active Pharmaceutical Ingredient
PBS	Phosphate Buffer Solution
FESEM	Field Emission Scanning electron microscopy
IU	International Units
FRA	Frequency response analysis
AC	Alternating current
E_i	Initial potential
E_s	Switching potential
E_f	Final potential
Ppb	parts-per-billion
i_{pc}	Cathodic peak current
i_{pa}	Anodic peak current
E_{pc}	Cathodic peak potential
E_{pa}	Anodic peak potential
KCl	Potassium Chloride
ATR	Attenuated Total Reflectance
MIC	Minimum inhibitory concentration

CHAPTER I

INTRODUCTION

CHAPTER I: INTRODUCTION

1.1 Background

Around 2016, one of my 7 years old nephew was admitted to the hospital due to recurrent severe fever. Based on the symptoms and some test results, doctor prescribed an antibiotic¹⁻² injection to him. But even after administering multiple doses (2 to 3 days), fever was not controlled and no improvement was observed. My nephew's condition got worse as if everybody thought it would be very difficult to recover and we were helpless. After 3 days we got a blood culture³⁻⁴ report, it was identified that it was happening due to an infectious microorganism presence in blood⁵. Another surprising identification was antibiotic resistance^{1, 6} towards the prescribed drug. So, the prescribed antibiotic did not work properly and his condition got worsen as if he would die. Then, immediately the doctor changed the antibiotic based on the culture report which is sensitive. Magically, his condition started improving and after a few days he recovered fully.

That incident shocked me badly! Doctors are also helpless in these situations. We got the blessings after the discovery of antibiotics by Alexander Flemming⁷ but we can't use it effectively in all cases due to the difficulties in diagnosis, specifically the long time needed for the incubation as a part of traditional microbiological test techniques³.

Since then, I started thinking about how to develop a test technique which can give an indication of antibiotic effectiveness within a few hours so that a doctor can prescribe the right antibiotic for the right patient.

1.2 Antibiotics

Among the drug molecules, a class of antimicrobial drugs used to treat infections caused by bacteria are known as antibiotics¹. Antibiotics work by killing bacteria or preventing their growth⁸⁻¹¹.

1.2.1 History of antibiotics: The lifesavers of modern medicine

The development of antimicrobial drugs marks a transformative milestone in medical science, fundamentally altering the management of infectious diseases and contributing significantly to global health¹²⁻¹³. Their unmatched efficacy in combating infections underscores their indispensable role in modern therapeutics¹⁴. Interestingly, the use of antibiotic-producing microorganisms dates back thousands of years, long before the discovery of bacteria¹⁵⁻¹⁶ as the cause of infections¹⁷. Ancient civilizations, including those in Serbia, China, Greece, and

Egypt, employed traditional remedies such as mouldy bread poultices to treat open wounds over 2,000 years ago¹⁸. The Eber's Papyrus, a medical document from 1550 BC, even lists mouldy bread and medicinal soil among its remedies¹⁸. While the widespread use of antibiotics is often associated with the modern "antibiotic era," evidence suggests their presence much earlier. Traces of tetracycline, for instance, have been found in human skeletal remains from 350–550 CE in Sudanese Nubia, indicating exposure to tetracycline-containing materials through diet^{9, 19}. Similarly, analysis of late Roman period skeletons from the Dakhleh Oasis revealed fluorochrome labeling consistent with tetracycline in their diet, further supporting the ancient use of such substances²⁰.

Moreover, the concept of antimicrobial intervention prior to the advent of modern antibiotics can be traced to age-old practices in traditional medicine, notably traditional Chinese medicine (TCM). A prominent illustration is the isolation of artemisinin; a powerful anti-malarial compound from artemisia species in the 1970s²¹. Historical records suggest that Chinese herbalists had employed artemisia based remedies for centuries to treat various ailments. Interestingly, artemisinin like compounds appear to be present in several other botanicals commonly used in TCM formulations^{21, 9}.

1.2.2 The discovery and evolution of antibiotics

In the late 19th century, scientists began exploring antibacterial chemicals. Paul Ehrlich, a German physician, discovered arsphenamine in 1909, effectively treating syphilis and pioneering "Chemotherapy"¹⁸. In 1928, Alexander Fleming accidentally discovered penicillin when *Penicillium Notatum* mold contaminated a *Staphylococcus* culture, inhibiting bacterial growth^{7, 22-24}. He isolated and purified penicillin, finding it highly effective and less toxic than disinfectants. After successful human trials, mass production began, aided by a British pharmaceutical company. Penicillin proved life-saving during a 1942 Boston fire and World War II, treating infections in soldiers and civilians, earning it the title "Wonder drug"²⁵. In 1942, Selman Waksman coined the term "Antibiotics," having discovered 27 during his research²⁶.

Early discoveries in antimicrobial therapy- in the late 19th century, scientists began exploring antibacterial chemicals²⁷. Paul Ehrlich, a German physician, observed that certain dyes selectively stained bacterial cells, leading him to hypothesize that chemicals could target specific bacteria without harming other cells⁹. In 1909, he discovered arsphenamine, a chemical effective against syphilis, marking the birth of "Chemotherapy"¹⁸.

Discovery of penicillin- in 1928, Alexander Fleming accidentally discovered penicillin when *Penicillium Notatum* mold contaminated a *Staphylococcus* culture, creating a bacteria-free zone. He isolated the mold, purified the active substance, and found it highly effective even at low concentrations. Penicillin is less toxic than contemporary disinfectants, became the first widely used antibiotic, saving countless lives. After successful clinical trials, mass production began with the help of a British pharmaceutical company²⁸. Penicillin proved crucial during World War II and a 1942 Boston fire, where it treated severe infections, earning it the title "wonder drug"²⁵.

Selman Waksman and the term "Antibiotics"- In 1942, Ukrainian-American microbiologist Selman Waksman coined the term "Antibiotics" and discovered 27 antibiotics during his research²⁶.

Pre-historic use of antimicrobials- ancient civilizations used natural remedies like herbs, honey, and moldy bread to treat infections. Evidence of tetracycline in ancient Nubian and Roman-era skeletons suggests early antibiotic use, though the source remains unclear.

Renaissance to enlightenment- Antonie van Leeuwenhoek's 1676 discovery of microorganisms laid the foundation for bacteriology². By the late 1800s, Robert Koch and Louis Pasteur linked bacteria to diseases, leading to experimental treatments like heavy metals for syphilis and gonorrhea, though these often-caused severe side effects².

Modern antimicrobial chemotherapy- The modern era began with Rudolf Emmerich and Oscar Low's discovery of Pyocyanase, derived from *Pseudomonas Aeruginosa*, in the late 19th century². Paul Ehrlich's work on dyes led to the discovery of salvarsan in 1909, the first modern antimicrobial agent. Ehrlich's collaboration with Robert Koch and Emil von Behring advanced immunology and antitoxin therapies, such as anti-streptococcal serum for endocarditis.

The end of the golden age- The rise of resistant gram-negative bacteria like *Acinetobacter* and *Pseudomonas* shifted focus back to older drugs like colistin and newer agents like tigecycline (2005)⁸. Recent developments include cephalosporins effective against MRSA (e.g., *ceftaroline*) and β -lactamase inhibitor combinations (e.g., ceftazidime/avibactam) for resistant strains².

1.2.3 How antibiotics work

The mechanism of action of antibiotics varies depending on their class and the target bacteria. Some antibiotics are specialized, targeting only specific bacteria, while others are broad-spectrum, effective against a wide range of bacteria. Antibiotics primarily work in two ways: they either kill bacteria through various mechanisms or inhibit their reproduction^{2, 29}. The major mechanisms include:

Disruption of cell membrane function: Certain antibiotics bind to bacterial cell membranes, increasing permeability and causing cellular contents to leak out, leading to bacterial death. Examples include polymyxins³⁰.

Inhibition of cell wall synthesis: Some antibiotics prevent bacteria from forming cell walls. Without a cell wall, bacteria lose their structural integrity and eventually die. Examples include penicillin and cephalosporins³¹.

Disruption of protein synthesis: Antibiotics like tetracyclines, macrolides, and aminoglycosides interfere with bacterial ribosomes, disrupting protein synthesis. This prevents the production of essential proteins needed for bacterial growth and replication³².

Inhibition of nucleic acid synthesis: Some antibiotics exert their effects by inhibiting key enzymes responsible for DNA replication and transcription, thereby obstructing bacterial proliferation and RNA synthesis³³. Representative classes with this mechanism include quinolones and rifamycins^{30, 34}.

Disruption of metabolic pathways: Certain antibiotics impair vital biochemical routes required for bacterial viability. For example, sulfonamides³⁵ and trimethoprim hinder the synthesis of folic acid, a crucial cofactor involved in nucleotide biosynthesis and DNA replication³⁶. Notably, antibiotics are designed to be selectively toxic, targeting bacterial cells without harming human cells.

1.2.4 The role of antibiotics

Antibiotics are applied for the treatment of a wide range of infections caused by bacteria like pneumonia, strep throat, urinary tract infections (UTIs), tuberculosis etc. It plays a very crucial role in modern medicine as the absence of antibiotics can cause massive deaths or severe illness. It works either by killing bacteria (bactericidal) or by inhibiting its growth (bacteriostatic) which allows the body's immune system to eliminate the infection³⁷. The

mode of action of antibiotic is termed as selective toxicity as it is designed in such a way which targets bacterial cells without harming human cells, exploiting differences in cellular structures and biochemical processes. The use of antibiotics should be appropriate to prevent the development of antibiotic-resistant which can make infections impossible or difficult to treat. It has the limitations as it is ineffective against viral infections³⁸.

1.2.5 Types of antibiotics

Antibiotics can be categorized according to their molecular architecture, mode of action, and range of antimicrobial efficacy³⁶. The following section outlines the principal classes and their defining characteristics:

Classification by chemical structure

Macrolides²⁹

Structure: Characterized by a large macrocyclic lactone ring:

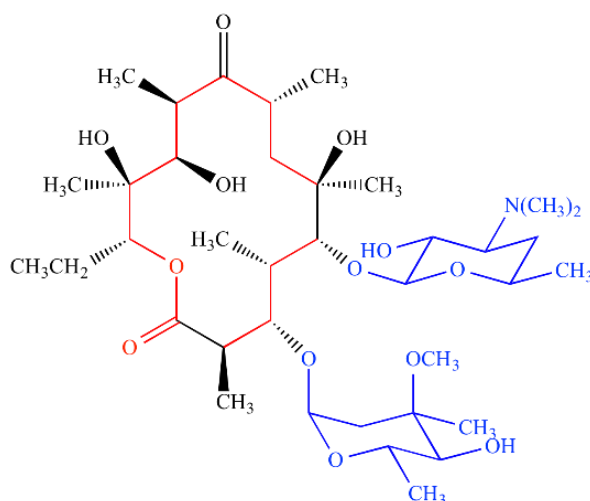


Figure 1. 1: Chemical structure of macrolides

Mechanism: Inhibit bacterial protein synthesis by binding to the 50S ribosomal subunit, disrupting peptide chain elongation.

Spectrum: Effective against gram-positive and some gram-negative bacteria.

Examples:

Erythromycin: Treats respiratory, skin, and sexually transmitted infections.

Azithromycin: Used for respiratory, skin, ear, and eye infections, as well as STIs.

Clarithromycin: A derivative of erythromycin with a broader spectrum of activity.

Tetracyclines³⁹⁻⁴⁰

Structure: Contain four cyclic rings:

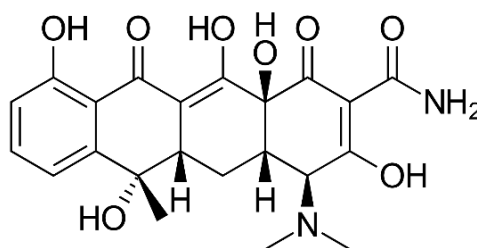


Figure 1. 2: Chemical structure of tetracyclines

Mechanism: Inhibit bacterial protein synthesis by binding to the 30S ribosomal subunit⁴¹.

Examples: Doxycycline.

Beta-lactams^{42, 7, 43}

Structure: Contain a beta-lactam ring:

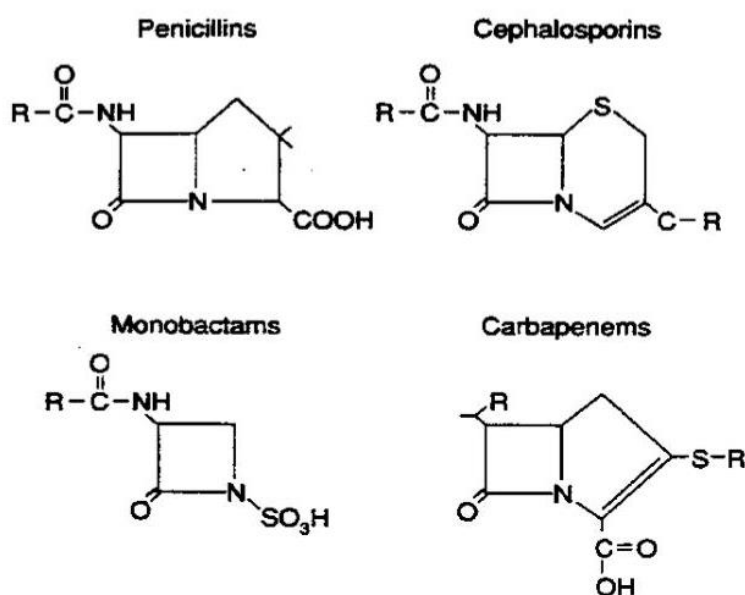


Figure 1. 3: Chemical Structure of β -lactams

Mechanism: Inhibit bacterial cell wall synthesis, leading to cell lysis.

Subclasses:

- (i) Penicillins²³ (e.g. Amoxicillin)
- (ii) Cephalosporins (e.g. Ceftriaxone)

(iii) Carbapenems (e.g. Meropenem)

(iv) Monobactams (e.g. Aztreonam)

Sulfonamides⁴⁴

Structure: Contain a sulfonamide group:

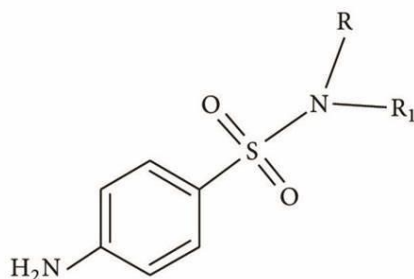


Figure 1. 4: Chemical structure of sulfonamides

Mechanism: Inhibit folic acid synthesis, essential for DNA production.

Example: Sulfamethoxazole.

Quinolones⁴⁵

Structure: Contain a quinolone core:

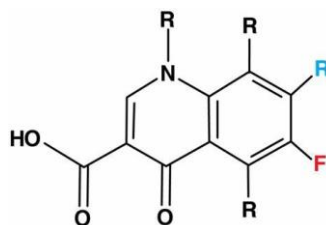


Figure 1. 5: Chemical structure of quinolones

Mechanism: Inhibit DNA gyrase and topoisomerase IV, preventing DNA replication.

Examples: Ciprofloxacin, Levofloxacin.

Aminoglycosides⁴⁶

Structure: Contain amino sugars linked to a hexose ring:

Mechanism: Bind to the 30S ribosomal subunit, disrupting protein synthesis.

Examples: Gentamicin, Streptomycin.

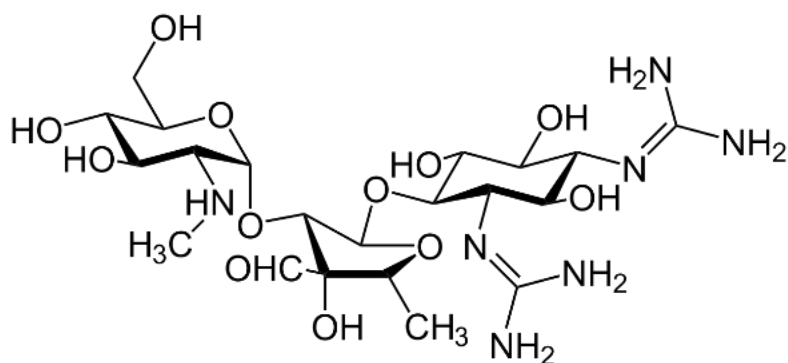


Figure 1. 6: Chemical structure of aminoglycosides

Classification by mechanism of action

Protein synthesis inhibitors⁴⁶

- (i) Aminoglycosides (e.g., Gentamicin)
- (ii) Tetracyclines (e.g., Doxycycline)
- (iii) Macrolides (e.g., Azithromycin)
- (iv) Chloramphenicol

Cell wall synthesis inhibitors⁴⁷⁻⁴⁸

- (i) Penicillins (e.g. Amoxicillin)
- (ii) Cephalosporins (e.g. Ceftriaxone)
- (iii) Carbapenems (e.g. Meropenem)
- (iv) Monobactams (e.g. Aztreonam)

Nucleic acid synthesis inhibitors^{45, 49}

- (i) Rifamycins (e.g., Rifampicin)
- (ii) Quinolones (e.g., Ciprofloxacin)

Metabolic pathway inhibitors⁴⁴

- (i) Sulfonamides (e.g., Sulfamethoxazole)
- (ii) Trimethoprim

Cell membrane disruptors³²

- (i) Polymyxins (e.g., Polymyxin B, Colistin)

Classification by spectrum of activity

Narrow-spectrum antibiotics

- (i) Target specific bacteria (e.g., Penicillin G for gram-positive bacteria).

Broad-spectrum antibiotics

- (i) Effective against a wide range of bacteria (e.g., Amoxicillin, Tetracycline, Ciprofloxacin).

Each antibiotic class targets specific bacterial infections and strains. Proper use of antibiotics is essential to ensure effectiveness and prevent resistance.

1.3 Antibiotic resistance

1.3.1 Antimicrobial resistance (AMR): A global crisis

Antimicrobial resistance (AMR) refers to the ability of microorganisms to persist and proliferate despite exposure to therapeutic agents intended to eliminate or suppress them^{50, 32, 18, 51-53}. Designated by the World Health Organization (WHO) as one of the ten most critical threats to global public health, AMR gained formal international recognition during the 2016 United Nations General Assembly, leading to the implementation of National Action Plans across numerous countries. By 2019, drug-resistant infections were linked to an estimated 4.95 million deaths worldwide, with the greatest impact observed in low- and middle-income nations, particularly within sub-Saharan Africa^{51, 54}. This exceeds deaths from tuberculosis, malaria, and HIV/AIDS combined⁵⁵. Without intervention, AMR could cause 10 million annual deaths by 2050⁵¹⁻⁵². This Special Issue by PLoS Medicine explores the complex nature of AMR and the challenges in designing equitable, cost-effective interventions to curb its spread^{52, 56-67}.

Drivers of AMR: The overuse of antibiotics is a major driver of AMR. Between 2000 and 2015, global antibiotic use rose by 65%, with LMICs contributing significantly^{68, 53}. Bacteria rapidly exchange DNA, often acquiring resistance mechanisms with minimal cost. AMR spreads across humans, animals, and the environment, making it a One-Health issue⁶⁹⁻⁷⁰. In LMICs, poor access to clean water, sanitation, healthcare, and affordable diagnostics exacerbates the problem. Addressing AMR requires coordinated efforts across human, agricultural, and environmental sectors⁷¹⁻⁷².

AMR in low and middle income countries: A study of 109 bloodstream infections in LMIC hospitals linked WHO-priority pathogens to higher mortality, longer hospital stays, and increased costs⁷³⁻⁷⁴. Despite the significant burden, data gaps hinder policy implementation. Analyses from 99 countries estimate 136 million annual hospital-associated resistant

infections, with China, Pakistan, and India bearing the highest burden⁷⁵. These findings highlight the urgent need for strategies to combat resistance in healthcare settings^{5, 76-77}.

AMR in high income settings: In high-income countries, antibiotic misuse has led to "superbugs" like methicillin-resistant *Staphylococcus aureus* (MRSA)⁷⁸. Antibiotic stewardship programs aim to reduce resistance by shortening treatment durations⁷⁹. However, the impact varies based on bacterial and antibiotic characteristics. Modeling studies suggest that shorter treatments may either increase or decrease resistance colonization, depending on context^{5, 80}.

AMR and healthcare facilities: Healthcare settings are hotspots for AMR transmission, often due to inadequate infection control. During the COVID-19 pandemic, surges in cases disrupted standard practices, increasing AMR colonization by 14%. However, stricter infection control measures reduced patient acquisition of resistant bacteria by 28%, underscoring the importance of robust protocols.

Global Response to AMR- In September 2024, a high-level UN meeting addressed AMR, proposing an enhanced framework to combat the crisis. Global leaders must prioritize investment in vaccines, diagnostics, and reporting systems. The COVID-19 pandemic highlighted the interconnectedness of global health and economies, with AMR projected to cause 10 million annual deaths and \$100 trillion in economic losses by 2050 if unaddressed^{50, 81-82}.

World AMR Awareness Week (WAAW): From November 18–24, 2024, the world celebrated World AMR awareness week under the theme "Educate. Advocate. Act now." This annual campaign aims to raise awareness, promote best practices, and unite stakeholders across human, animal, plant, and environmental health sectors to combat AMR. The theme was chosen based on global stakeholder feedback, emphasizing the need for immediate action⁸³.

1.3.2 Causes of antibiotic resistance

Antibiotic resistance arises when bacterial populations undergo adaptive changes in response to antibiotic exposure, diminishing or nullifying the therapeutic efficacy of these drugs⁸⁴⁻⁸⁵. This phenomenon happens due to several factors⁵³:

- (i) Overuse of the antibiotics
- (ii) Inappropriate prescribing

- (iii) Antibiotic residues from agricultural use⁸⁶
- (iv) Availability of few new antibiotics
- (v) Regulatory barriers

1.3.3 Mechanism of antibiotic resistance

Antibiotic resistance emerges when bacteria acquire mechanisms that enable them to withstand the effects of drugs intended to eliminate them. This resistance can manifest through various strategies, including^{53, 58, 87-89}:

- (i) Reducing the intracellular uptake of the antibiotic
- (ii) Altering the molecular target to diminish drug binding⁹⁰
- (iii) Chemically modifying or degrading the antibiotic
- (iv) Actively expelling the drug via efflux pumps

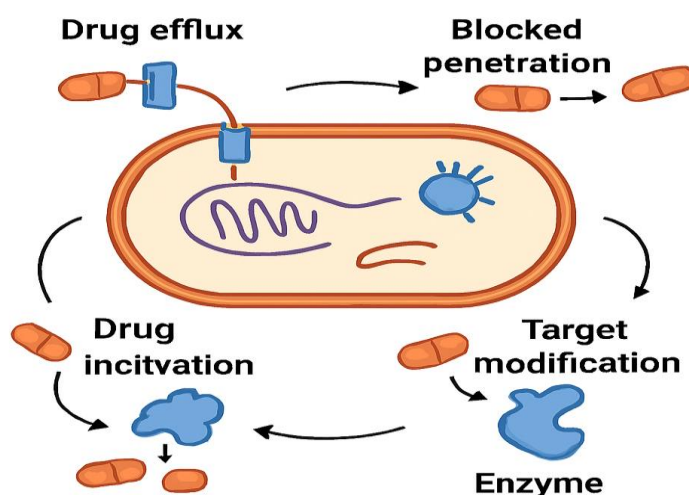


Figure 1. 7: Examples of mechanism of antimicrobial resistance⁸²

Modification of target sites: Bacteria can modify the sites where antibiotics bind, making the drugs ineffective. For example, mutations in the bacterial ribosomal proteins can prevent macrolides from binding effectively⁸⁹.

Enzymatic degradation of antibiotics: Some bacteria generate enzymes that degrade the antibiotic, preventing it from causing damage⁹¹. For example, beta-lactamase enzymes degrade beta-lactam antibiotics like penicillin and cephalosporins⁹².

Formation of biofilm: Bacteria can create biofilms intricate communities of bacteria surrounded by a protective matrix. Those biofilms can block antibiotics from penetrating and protect the bacteria from the immune system.

Efflux pumps: Certain bacteria have efflux pumps that actively expel antibiotics, reducing the drug's concentration within the cell to non-lethal levels.

Reduction of permeability: Some bacteria can reduce the permeability of their cell membranes to certain antibiotics, hindering the drug's ability to penetrate the bacterial cell.

Horizontal gene transfer mechanism: Horizontal gene transfer (HGT) mechanism is a fascinating process in which an organism transfers genetic materials to a different organism which is not its offspring. That subject process plays a significant role in the evolution of organisms like bacteria. The primary mechanisms through which horizontal gene transfer occurs are transformation, conjugation and transduction. These mechanisms enable bacteria to acquire new genes⁹³. Including those for antibiotic resistance, virulence factors, and metabolic capabilities. It can significantly enhance their adaptability and survival in various situations⁹⁴.

1.3.4 Examples of antibiotic resistance

Methicillin-resistant *Staphylococcus aureus*: It (MRSA) is a bacterial strain that exhibits resistance to methicillin and other β -lactam antibiotics, posing significant challenges in clinical management. It is associated with a range of infections, from localized skin conditions such as painful, purulent lesions to more serious systemic manifestations including pneumonia, septicemia, and osteoarticular infections^{43, 50}.

Causes of resistance:

- (i) Overuse and misuse of antibiotics⁹⁵⁻⁹⁶
- (ii) Spread in hospitals and healthcare settings
- (iii) Community transmission in crowded or unhygienic conditions

Vancomycin-resistant enterococci (VRE): VRE are bacteria resistant to vancomycin, commonly found in the intestines and female genital tract. They cause infections that are challenging to treat due to limited effective antibiotics⁹⁷.

Multi-drug-resistant tuberculosis (MDR-TB): It refers to the infections caused by mycobacterium tuberculosis strains that exhibit resistance to at least two primary anti-

tubercular agents isoniazid and rifampicin. This resistance significantly hinders both therapeutic management and public health control strategies⁹⁸. MDR-TB is caused by mycobacterium tuberculosis resistant to at least two first-line anti-TB drugs, isoniazid and rifampicin, complicating treatment and control efforts.

1.3.5 Impact of antibiotic resistance on healthcare

Public Health Impacts-

- (i) Increased mortality: Resistant infections are harder to treat, leading to higher death rates.
- (ii) Longer hospital stays: Patients with resistant infections often require extended hospitalization, increasing complications.
- (iii) Limited treatment options: Common infections and medical procedures become riskier due to fewer effective antibiotics⁹⁹.

Economic Impacts

- (i) Higher healthcare costs: Treating resistant infections requires more expensive and potent antibiotics¹⁰⁰.
- (ii) Global economic burden: The World Bank estimates antibiotic resistance could cost the global economy up to \$3.4 trillion annually by 2030¹⁰¹.

Broader implications

- (i) Public health threat: Resistant bacteria can spread rapidly, posing a widespread health risk.
- (ii) Global health emergency: Untreatable infections could lead to global crises, increasing insecurity and instability.

1.3.6 Global threats of antibiotic resistance

Antibiotic resistance is a major global health crisis, posing severe risks to public health and safety. Currently, it is responsible for approximately 1.27 million deaths each year, a figure that could escalate to 39 million by 2050 if no action is taken. The overuse and misuse of antibiotics have led to the rise of multidrug-resistant organisms, commonly known as "superbugs," which are resistant to multiple antibiotics and complicate treatment¹⁰². The economic impact is equally alarming, with the World Bank projecting potential global GDP losses of up to \$3.4 trillion annually by 2030 due to antibiotic resistance^{50, 103-106}.

1.3.7 Strategies to combat antibiotic resistance

Antibiotic resistance is an escalating public health challenge, and addressing it requires a comprehensive understanding of its mechanisms. Key strategies include⁵⁰⁻⁵¹:

Antibiotic stewardship: Promoting the rational and judicious use of antibiotics to reduce unnecessary consumption¹⁰⁷.

Research and development: Investing in the discovery of new antibiotics and alternative therapies to combat resistant infections¹⁰⁸⁻¹⁰⁹.

Infection control: Enforcing stringent infection prevention measures in healthcare facilities to limit the spread of resistant bacteria¹¹⁰⁻¹¹¹.

1.4 Determination of antibiotics

1.4.1 Types of methods for determination of antibiotics

The qualitative and quantitative determination of antibiotics involves different methods to identify and measure the presence of antibiotics in various samples.

Qualitative determination: It is used to identify the presence or absence of specific antibiotics in a sample. The common techniques such as thin-layer chromatography (TLC), paper chromatography (PC), UV-visible spectrophotometer, high-performance liquid chromatography (HPLC), and mass spectrometry (MS) are commonly used for qualitative analysis^{112, 36}.

Quantitative determination: Quantitative determination techniques are used to measure the concentration of antibiotics present in a sample. The common techniques such as UV-visible spectrophotometer, high-performance liquid chromatography (HPLC), gas chromatography (GC), potentiometry, and mass spectrometry (MS) are used for quantitative analysis. Besides chemical methods, microbiological methods are also used for quantitative determination of antibiotics which is known as microbiological assay or bioassay^{36, 112-113}.

1.4.2 Chemical methods

Several chemical methods are used for qualification and quantitation of antibiotics. The most common techniques are UV-visible spectrophotometer, high-performance liquid chromatography (HPLC), gas chromatography (GC), potentiometry, and mass spectrometry (MS)^{36, 45, 114}.

1.4.3 Microbiological methods

Microbiological assays, also known as bioassays, are analytical techniques used to evaluate the relative potency of a compound by determining the quantity required to elicit a defined biological response in a standardized test organism. This approach relies on comparing the inhibitory effects of different concentrations of the test antibiotic against microbial growth with those produced by a reference antibiotic of established activity. Among the commonly employed methodologies are the cylinder-plate (or cup-plate) method and the turbidimetric (or tube assay) method^{113, 115}.

1.5 Microbial enumeration of antibiotics resistance

1.5.1 Methods of detection of antibiotic resistance

Determination of antibiotic resistance is very crucial for effective treatment and to control infection^{32, 116-117}. These are the common methods are usually used for detecting antibiotic resistance-

Phenotypic methods^{3, 36}

Disk diffusion method (Kirby-Bauer Test): This method measures the zone of inhibition around antibiotic-impregnated disks on an agar plate.

Broth dilution method: This technique determines the minimum inhibitory concentration (MIC) by observing bacterial growth in serial dilutions of antibiotics.

Agar dilution method: This method is similar to broth dilution but performed on agar plates.

E-test (Epsilometer Test): In E-Test use a strip with a gradient of antibiotic concentration to determine the MIC¹¹⁵.

Molecular methods

Polymerase chain reaction (PCR): In this technique detects specific antibiotic resistance genes by amplifying DNA sequences¹¹⁸.

DNA microarray: Screens for multiple resistance genes simultaneously using DNA probes³.

Whole-genome sequencing (WGS): Provides comprehensive genetic information, identifying resistance genes and mutations³.

Metagenomics: Analyzes genetic material from environmental samples to detect resistance genes¹¹⁵.

Advanced technologies

Matrix-assisted laser desorption/ionization: Time-of-flight mass spectrometry (MALDI-TOF MS) is a proteomic technique used to identify bacterial species and detect resistance-associated biomarkers by analyzing characteristic protein signatures.

Automated systems: Utilizes advanced technology for rapid and accurate susceptibility testing¹¹⁵.

Table 1.1: Different methods of antimicrobial susceptibility testing and tentative time

Phenotypic Methods	Time	Molecular-based Methods	Time	Mass Spectrometry	Time
Dilution	48 h	PCR	Several hours	MALDI-TOF	Several hours
Diffusion	48 h	qPCR		MS	
Gradient test	48 h	Cycle sequencing	24 – 48 h		
Chromogenic media	24 h	NGS	Several days		
Automated devices	20 h				

1.5.2 Traditional methods

Traditional methods for detecting antibiotic resistance primarily involve phenotypic tests that observe the growth response of bacteria to antibiotics^{115, 119}. Here are some commonly used traditional methods:

Disk diffusion method (Kirby-Bauer Test)³

Principle: Antibiotic-impregnated disks are placed on an agar plate inoculated with the test organism.

Measurement: The zone of inhibition (clear area) around the disk is measured to determine susceptibility. Larger zones indicate greater sensitivity, while smaller or no zones indicate resistance.

Broth dilution method³

Principle: Serial dilutions of antibiotics are added to a broth containing the test organism.

Measurement: The minimum inhibitory concentration (MIC) is determined as the lowest concentration that inhibits visible bacterial growth after incubation.

Agar dilution method³

Principle: Similar to broth dilution, but performed on agar plates. Serial dilutions of antibiotics are mixed with agar, and bacterial inocula are spot-inoculated on the surface.

Measurement: The MIC is determined by observing the lowest concentration of antibiotic that prevents visible growth of the bacteria.

E-test (Epsilometer test)³

Principle: A strip with a gradient of antibiotic concentration is placed on an inoculated agar plate.

Measurement: The MIC is read at the point where the bacterial growth edge intersects the strip's scale.

Automated systems³

Principle: Automated systems like the VITEK, Phoenix, and MicroScan perform susceptibility testing using advanced technology to provide rapid and accurate results.

Measurement: These systems provide MIC values and categorize bacteria as susceptible, intermediate, or resistant.

1.5.3 Drawbacks of traditional methods

Considering the accuracy, reliability and cost traditional methods are being used mostly. Though traditional methods are widely used for detecting antibiotic resistance but it has several drawbacks¹¹⁶. Here are some key limitations³-

Time-consuming: Incubation or culture-based methods: Traditional methods like disk diffusion and broth dilution require bacterial incubation and culture which takes long time like 24 to 48 hours or longer. It delays the diagnosis process and the treatment decisions by the doctors as well.

Labor-intensive: Mostly manual activities or processes: It is often required manual preparation and interpretation which increase the workload for laboratory personnel and the risk of human error.

False negative results due to limited sensitivity: Detection limits: Some traditional methods may not detect low levels of antibiotic resistance or emerging resistance mechanisms which potentially can lead to false-negative results.

Variability in data or results: Inconsistency: Inconsistent data can generate due to differences in technique, media, incubation conditions, and interpretation which can lead to potential discrepancies between laboratories or personnel or lots of reagents.

Resource-intensive: Equipment and reagents: Traditional methods require specific equipment, reagents, and well-trained personnel as mostly manual works. It might not be accessible in all settings especially in resource-limited regions.

Inadequacy of genetic insight: Phenotypic focus: Subject traditional methods focus on the phenotypic expression of resistance and do not provide information about the genetic basis of resistance, which is important for understanding the transmission and evolution.

Limitations of traditional methods for studying antimicrobial resistance

Traditional in vitro assays for studying antimicrobial resistance (AMR) often fail to replicate clinical conditions, leading to insights that may lack clinical relevance. Key limitations include:

Non-physiological conditions: Culture media differ significantly from human body fluids. Bacteria are exposed to antibiotics for shorter durations than in actual treatment courses. Fluctuations in drug concentrations, which promote resistance mutations, are rarely modeled.

Biofilm vs. planktonic states: Most assays use planktonic bacteria, while chronic infections often involve biofilms. Bacteria in biofilms exhibit different resistance mechanisms and genetic regulators compared to planktonic states. For example, *Pseudomonas aeruginosa* shows distinct genetic loci influencing resistance in each state.

Inoculum effect: Standard assays like minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) ignore the impact of initial bacterial load on antibiotic potency. Even small changes in MIC (e.g., ≤ 2 -fold or ≤ 4 -fold) can have significant clinical implications, despite being dismissed as measurement errors.

Discrepancies between In Vitro and In Vivo results: Insights from in vitro studies often differ from in vivo findings. For instance, mutations in *Mycobacterium tuberculosis* affecting

isoniazid resistance in mice showed no effect in vitro.

Time constraints: Traditional methods typically require 16–20 hours, sometimes exceeding 48 hours. Delays often lead physicians to prescribe broad-spectrum or multiple antibiotics without testing, exacerbating resistance. These limitations highlight the need for more clinically relevant and rapid diagnostic tools to address AMR effectively¹¹⁶.

1.6 Recent researches

1.6.1 Review of recent research approaches to develop alternate testing technique

Recent advancements in diagnostic technologies are transforming AMR detection, offering faster, more accurate, and comprehensive methods. Many research articles published recently show scientist are trying to develop alternative ways to diagnose antibiotic resistance. Key techniques include:

Whole genome sequencing (WGS)³: Sequences the entire genome of microorganisms to identify resistance genes and mutations¹²⁰. Provides detailed genetic insights into resistance mechanisms.

MALDI-TOF MS (Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry)³⁶: Analyzes microbial protein profiles for species identification and resistance markers. Enables rapid and accurate detection, ideal for clinical use.

Flow cytometry: Uses laser-based technology to analyze cell characteristics. Offers high-throughput screening and detailed microbial population analysis.

Microfluidics: Integrates lab processes on a single chip for rapid, automated testing. Reduces sample volume and processing time through miniaturization.

Biosensors: Utilizes biological components (e.g., enzymes, antibodies) to detect resistance markers. Provides high sensitivity and specificity, with potential for point-of-care testing¹²¹.

Immunodiagnostic techniques: Employs antibodies to detect resistance-associated antigens. Enables rapid and specific detection, suitable for clinical diagnostics.

Luminescence-based assays: Uses luminescent markers to detect microbial growth and resistance. Offers high sensitivity and rapid results.

Colorimetric assays: Relies on color changes to indicate resistance markers. Simple,

cost-effective, and suitable for field testing. While these advanced techniques significantly improve AMR detection, challenges remain in identifying resistance prior to initiating treatment. The only pitfall is time.

1.6.2 Electrochemical approaches for detecting drug resistance

Electrochemical methods have gained attention for understanding and detecting drug resistance in microorganisms, particularly through the study of drug compounds and efflux pump proteins^{122, 38-39}.

Advantages of electrochemical sensors:

- (i) High sensitivity: Capable of detecting low analyte concentrations.
- (ii) Rapid analysis: Provides quick results, ideal for time-sensitive applications.
- (iii) Complex sample compatibility: Can analyze biological samples like blood or urine.
- (iv) Cost-effectiveness: Inexpensive and portable, with potential for point-of-care use.

While no electrochemical sensor for point-of-care antibiotic resistance detection has been developed yet, these sensors are widely used in environmental monitoring, biotechnology, and industrial process control¹²³⁻¹²⁴. Their effectiveness has spurred research into medical applications, including drug resistance analysis.

Key research focus:

- (i) Quantitative determination of antibiotics
- (ii) Recovery study in blood sample
- (iii) Simulation of study with *E. coli* ATCC strain and β -lactamase
- (iv) Electrochemical methods offer promising avenues for advancing drug resistance detection and understanding antibiotic mechanisms.

1.7 Objectives of the research work

1.7.1 Objectives

The fundamental objective of this project is to seek a rapid, sensitive, and cost-effective electrochemical testing technique which can facilitate early diagnosis of antibiotic resistance of those antibiotics which mechanism of action indicates the inactivation or breakdown of antibiotic molecule upon interaction with that bacterium. Considering the mechanism of action of antibiotics, antibiotic molecules are selected from the cephalosporin group and quinolone group.

Cephalosporin antibiotics are having β -lactam ring. These antibiotics work by inhibiting or disrupting cell wall synthesis. The mechanism is, antibiotic molecule binds to penicillin-binding proteins (PBPs) in bacterial cell walls which results bacterial death. In this mechanism, a certain quantity of antibiotic is degraded to kill the bacteria and the remaining quantity of antibiotic is excreted from the body. On the other hand, resistant bacteria have the capability to degrade antibiotic molecules by generating β -Lactamase enzyme^{31, 51}. So, the quantitative determination of antibiotics over a period of time, after taking or administering antibiotics can give an idea about the nature of bacteria whether the bacteria are resistant or sensitive. Because, if any bacteria are sensitive against a specific antibiotic, then, the bacteria will be killed by the antibiotic. A very small portion of antibiotic from administered quantity will be degraded for the killing of bacteria and the remaining quantity of antibiotic can be determined. If the bacteria are resistant then, the concentration or peak response will decay gradually with respect to the time and continuous growth of bacteria. β -lactamase enzyme was taken into consideration to conduct these studies with cephalosporin antibiotics.

Besides, to see the suitability of electrochemical methods on detection of antibiotic resistance other than β -lactams, selected another molecule from quinolone group which is effective against both gram-positive and gram-negative bacteria¹²⁵. The mechanism is also different. Its mechanism of action involves the suppression of bacterial DNA gyrase and topoisomerase IV enzymes that are critical for the processes of DNA replication and transcription. Quinolones passively diffuse through porins in the gram-negative outer membrane and once inside, it accumulates due to interactions with cytoplasmic components^{49, 45}. A suitable gram-negative bacteria strains *E. coli* ATCC 8739^{126, 127} was selected to conduct this study.

This novel diagnostic technique aims to provide real-time detection of resistant bacterial strains in biological samples like blood or urine. The detection would be through the identification of specific resistance markers. The technique will leverage the high sensitivity and specificity of electrochemical sensors, with the goal of improving patient outcomes by facilitating timely and accurate treatment decisions. Additionally, this project seeks to enhance the understanding of the interactions between antibiotics and microbial resistance mechanisms, ultimately contributing to the development of more effective therapeutic strategies. Furthermore, this research will establish a foundational framework that fosters innovative thinking toward the development of targeted methodologies. These approaches hold the potential for broad applicability in detecting diverse drug-resistant bacterial strains,

thereby offering a robust experimental basis for optimizing antibiotic use and elucidating resistance mechanisms.

The core objectives are summarized briefly:

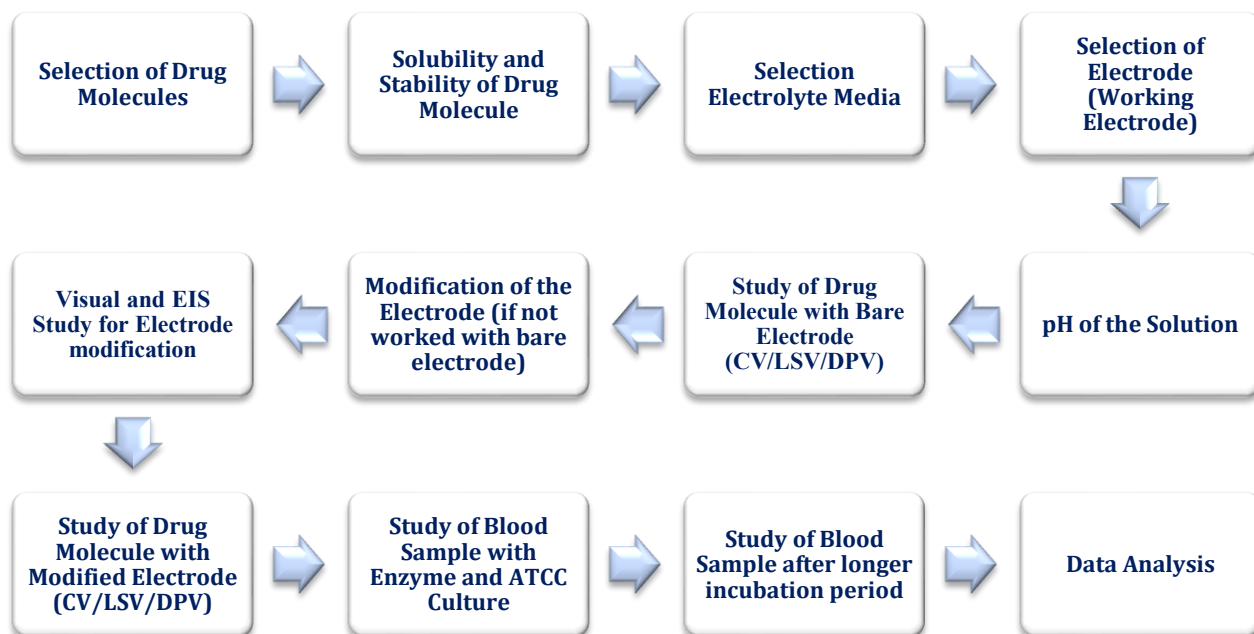
- (i) Development of electrochemical method which can detect antibiotics from blood sample.
- (ii) Simulated study using cephalosporin antibiotics using β -lactamase enzyme and quinolone molecules using *E. coli* strain ATCC 8739.
- (iii) The objective of the research is to develop an alternative rapid diagnosis electrochemical technique for the group of antibiotics having mechanism of inactivation or breakdown of antibiotics which will take less time than the traditional test methods.
- (iv) The capacity to swiftly detect antibiotic resistance holds immense potential. Developing an efficient and rapid diagnostic system would represent a major advancement in guiding appropriate antibiotic use and curbing the emergence of drug-resistant pathogens.
- (v) It would be environment friendly, trace amount detection and cost effective.
- (vi) If diagnosis can be performed immediately then doctors can select the right antibiotic for the right patient.
- (vii) Suitable pH range detectability

1.7.2 Opportunities

- (i) If any rapid detection technique can be developed then the resistance as well as sensitivity/ reactivity can be diagnosed instantly or within shortest possible of time.
- (ii) If diagnosis can be performed immediately then doctors can select the right antibiotic for the right patient.
- (iii) Critical patients can be prescribed properly
- (iv) Antibiotic resistance can be prevented
- (v) Millions of lives can be saved

1.8 Overview of present work

1.8.1 Working flow chart



1.8.2 Experimental details

- (i) Detail electrochemical characterization of the proposed antibiotics has been carried out by using cyclic voltammetry, linear swipe voltammetry and differential pulse voltammetry.
- (ii) The same characterizations have been carried out in Blood samples in presence of β -lactamase enzyme and bacteria strain culture.
- (iii) All of the above studies have been performed in solution phase and maintaining with a physiological pH.
- (iv) A comparative study has been done based on the data obtained with different time span up to 24 hours which facilitates to draw an inference about the effectiveness of antibiotics as well as incubation for longer period like traditional microbiological methods.

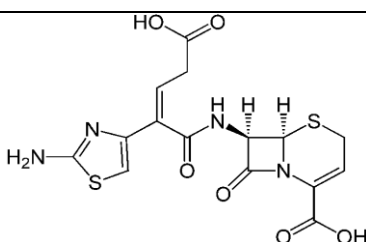
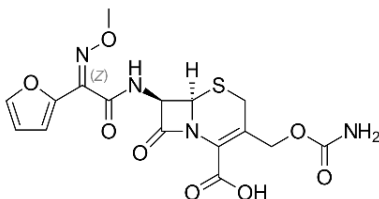
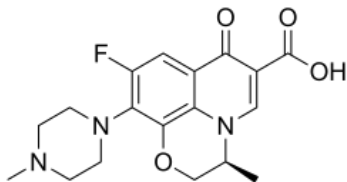
1.8.3 Selection of drug molecules

Different group or class of antibiotics have been taken into consideration for the research. Among which there was a special focus on β -lactam antibiotics as a large number drugs can be studied with the same principle. Also, taken into consideration other groups like fluoroquinolone. Brief details of the drug molecules are described below-

Antibiotic molecules ceftibuten and cefuroxime were selected from the cephalosporin group. Both antibiotics are having β -lactam ring. These antibiotics work by inhibiting or disrupting cell wall synthesis. The mechanism is, antibiotic molecule binds to penicillin-binding proteins (PBPs) in bacterial cell walls which results bacterial death⁴³.

On the other hand, to see the suitability of electrochemical methods on detection of antibiotic resistance other than β -lactams, selected another molecule levofloxacin from quinolone group which is effective against both gram-positive and gram-negative bacteria. The mechanism is also different. It works by inhibiting bacterial DNA gyrase and topoisomerase IV, which are essential for DNA replication and transcription. Levofloxacin passively diffuses through porins in the Gram-negative outer membrane and once inside, it accumulates due to interactions with cytoplasmic components.

Table 1.2: Brief details of the drug molecules

Sl	Name of Drug Molecules	Structure	Class of Drug	Details of Activity
1	Ceftibuten		3 rd generation cephalosporin β -lactam antibiotics	Exhibits extensive antimicrobial activity, targeting a wide range of gram-negative bacteria along with specific gram-positive species
2	Cefuroxime Sodium		2 nd generation cephalosporin	It exhibits broad-spectrum activity against both gram-positive and gram-negative organisms
3	Levofloxacin		Fluoroquinolone	Broad-spectrum antibiotic of the fluoroquinolone drug class

1.8.4 Techniques used

Several electrochemical techniques were used for the qualitative and quantitative determination. Detailed electrochemical characterization of the proposed antibiotics has been carried out by using Cyclic Voltammetry, Linear Sweep Voltammetry and Differential Pulse Voltammetry.

In the research study, analyzed the redox behavior of β -lactam antibiotics, fluroquinolone and macrolides using different supporting electrolytes and tried to develop a method a simple and effective process to determine these antibiotics selectively in presence of another in binary mixtures. Various electrochemical techniques such as cyclic voltammetry (CV), differential pulse voltammetry (DPV), linear sweep voltammetry (LSV) were used for study. Theory and principles are described briefly in below-

1.8.5 Electrochemistry as a tool for analysis

One of the primary categories of instrumental analysis is electroanalysis. Electroanalytical methods utilize electrically conductive probes, known as electrodes, to make contact with the analyte solution. These electrodes, when used with electric or electronic devices, measure an electrical parameter of the solution. This measured parameter correlates with the identity or quantity of the analyte in the solution.

Electroanalytical methods are categorized based on the electrical parameters they measure. The main electroanalytical techniques include potentiometry, amperometry, conductometry, electrogravimetry, voltammetry (and polarography), and coulometry. These methods are named according to the specific electrical properties they measure. Potentiometry assesses electric potential (voltage) while maintaining a constant (usually nearly zero) electric current between the electrodes. Amperometry measures electric current (amperes) while keeping the potential constant. Conductometry evaluates conductance (the ability of a solution to carry an electric current) while a constant alternating-current (AC) potential is maintained between the electrodes. Electrogravimetry is a gravimetric technique where the solid to be weighed is deposited on one of the electrodes. Voltammetry varies the potential in a controlled manner while monitoring the current. Polarography, a subtype of voltammetry, uses a liquid metal electrode. Coulometry measures the amount of electricity (coulombs) consumed during an electrochemical reaction involving the analyte. Most electroanalytical methods depend on the flow of electrons between the electrodes and the analyte. The analyte must be able to either

accept electrons (reduction) from the electrode or donate electrons (oxidation) to the electrode¹²⁸.

The primary electrochemical techniques used for detecting antibiotics include cyclic voltammetry, linear sweep voltammetry, differential pulse voltammetry, chronocoulometry, and chronoamperometry.

Electrochemistry has emerged as a powerful tool widely used to address various challenges in fields such as organic chemistry, biochemistry, materials science, and environmental science. Many natural and biochemical processes involve redox reactions, and their mechanisms can be easily elucidated through voltammetry experiments. For instance, cyclic voltammetry is the most commonly used modern electro-analytical method for investigating the mechanisms of redox systems.

1.8.6 Cyclic voltammetry (CV)

Cyclic voltammetry (CV) is a widely used and versatile electroanalytical technique for studying redox reactions. It involves scanning the electrode potential to identify and characterize redox couples based on peak potentials and scan rate variations.

Key Features of CV: Mechanistic Insights-Provides information on electron transfer mechanisms and adsorption behavior of reactants/products on the electrode surface¹²⁹.

Experimental Setup: A stationary electrode is immersed in an unstirred solution, and a triangular potential scan (1 mV/s to hundreds of mV/s) is applied. The resulting current vs. potential plot, called a voltammogram, reveals redox peaks.

Applications: Often the first experiment in electrochemical studies. Operates under diffusion-controlled conditions, making it ideal for analyzing redox systems.

CV is a powerful tool for understanding electrochemical reactions and is widely used due to its simplicity, effectiveness, and ability to provide detailed mechanistic insights.

1.8.7 Linear sweep voltammetry (LSV)

Linear sweep voltammetry (LSV) is an up-front electrochemical technique. Similar to cyclic voltammetry, LSV involves a linear sweep of potential; however, it only sweeps in one direction, from the lower potential limit to the upper potential limit, rather than cycling back and forth. This method is especially useful for irreversible systems where a reverse sweep

would not provide additional information¹³⁰⁻¹³¹.

1.8.8 Differential pulse voltammetry (DPV)

Differential pulse voltammetry (DPV) is frequently used for electrochemical measurements. It is a variant of linear sweep voltammetry or staircase voltammetry, where a series of regular voltage pulses are superimposed on the potential linear sweep or stair steps. The current is measured immediately before each potential change, and the difference in current is plotted as a function of potential. By sampling the current just before the potential change, the effect of the charging current is minimized¹³².

In DPV, a series of voltage pulses are applied to the working electrode in a stepwise manner. These pulses consist of a fixed potential increment (pulse height) followed by a short relaxation period. During each pulse, the current response, which is proportional to the analyte concentration in the solution, is recorded.

The key advantage of DPV is its ability to separate the faradaic current response of the analyte from the background current caused by other interfering species or electrode processes. This is achieved by applying a small potential increment during each pulse, allowing for the discrimination of the faradaic current related to the analyte's oxidation or reduction process. By measuring the current difference between the initial and final potentials of each pulse, the background current is effectively canceled out, resulting in an improved signal-to-noise ratio and enhanced sensitivity.

DPV offers several advantages as an electrochemical technique. It provides excellent resolution and can detect low concentrations of analytes with high sensitivity. The technique is relatively fast, enabling rapid sample analysis. Additionally, DPV is versatile and applicable to a wide range of electroactive species, making it suitable for various applications in environmental analysis, pharmaceutical research, and food testing¹³²⁻¹³⁴.

Uses of DPV

These measurements are valuable for studying the redox properties of extremely small amounts of chemicals due to the following two features:

- (i) In these measurements, the effect of the charging current can be minimized, so high sensitivity is achieved.
- (ii) Faradaic current is extracted, so electrode reactions can be analyzed more precisely.

1.8.9 Electrochemical impedance spectroscopy

Electrochemical impedance spectroscopy (EIS) provides kinetic and mechanistic insights into various electrochemical systems and is widely utilized in fields such as corrosion studies, semiconductor science, energy conversion and storage technologies, chemical sensing, biosensing, and noninvasive diagnostics. EIS operates by perturbing an electrochemical system in equilibrium or steady state using a sinusoidal signal (AC voltage or AC current) across a wide range of frequencies and monitoring the system's sinusoidal response (current or voltage)¹³⁵.

Assuming the electrochemical system under study is a linear time-invariant system (where the output signal is linearly related to the input signal and the system's behavior remains constant over time), EIS functions as a "transfer function" technique, modeling the output signal (AC current or AC voltage) in relation to the input signal (AC voltage or AC current) across various frequencies. EIS's significance over other electrochemical techniques lies in its ability to differentiate and provide comprehensive information on the various electrical, electrochemical, and physical processes occurring in a real electrochemical system.

This differentiation is challenging due to the diverse time behaviors (ranging from very fast to very slow) of these processes. For instance, the resistance of a liquid electrolyte, the differing bulk and grain boundary conductivities when using a solid polycrystalline electrolyte, the charging and discharging of the electric double layer at the electrolyte/electrode interface, the capacitive behavior of the electric double layer influenced by the electrode surface morphology and electrolyte composition, the kinetics of an electrode charge-transfer reaction, homogeneous reactions and adsorption/desorption phenomena coupled with the electrode charge-transfer reaction, and mass transfer phenomena (diffusion of species to the electrode surface) all exhibit different time constants (τ), which is a measure of a process's time behavior. The time constant of a process is defined as $\tau = RC$, where R is the resistance (in ohms) and C is the capacitance (in farads).

Note that the time constant is measured in seconds (s). $[(1 \text{ ohm}) \times (1 \text{ farad}) = (1 \text{ V}/1 \text{ A}) \times (1 \text{ coulomb}/1 \text{ V}) = 1 \text{ coulomb}/\text{ampere} = 1 \text{ s}]$. EIS measurements in an electrochemical system can be modeled as an equivalent electrical circuit, consisting of standard passive components (such as resistors, capacitors, and inductors) and more complex (distributed) elements, connected in various ways. Each process in the system can be represented by an equivalent electrical circuit characterized by a different time constant. To facilitate this, most

electrochemical analyzers come equipped with software that enables the simulation of impedance data to a model circuit. Specialized software for equivalent circuit modeling, such as Zview and Zplot (Scribner Associates, Inc.), is also available. Before simulating EIS data to an equivalent circuit, it is crucial to validate the data.

This can be done using the Kramers-Kronig test, which is available in most software provided with electrochemical analyzers. It is important to note that (i) there is no single model circuit for a given impedance spectrum, and (ii) the quality of the modeling improves with the number of components included in the circuit (the more, the better). Therefore, each component must be logically correlated to a specific process by scientific reasoning.

Key features of EIS:

- (i) Frequency Range: EIS can probe the system at different frequencies, typically ranging from millihertz to megahertz. This wide range allows it to capture both fast and slow processes.
- (ii) Non-destructive: EIS is generally a non-destructive technique, meaning it does not significantly alter the system being studied.
- (iii) Detailed analysis: EIS can separate different electrochemical phenomena, such as charge transfer resistance, double-layer capacitance, and diffusion processes.

Applications of EIS:

- (i) Battery Research: Understanding battery performance and degradation mechanisms.
- (ii) Corrosion Studies: Monitoring the corrosion behavior of metals and coatings.
- (iii) Sensor Development: Characterizing the performance of electrochemical sensors.
- (iv) Biomedical Devices: Studying the properties of biosensors and medical implants.

EIS data interpretation:

EIS data is typically represented in the form of Nyquist or Bode plots, which show the impedance as a function of frequency. These plots help in analyzing the various contributions to the overall impedance and in modeling the electrochemical system. EIS is a valuable tool in both fundamental research and practical applications, offering insights into a wide range of electrochemical systems¹³⁵.

Here, EIS technique was utilized to characterize the electrode surface post-modification to ensure that the conductive layer has increased its sensitivity and detection capabilities.

1.8.10 Fourier transform infrared (FT-IR) spectroscopy

FT-IR spectroscopy operates on the principle of molecular bond vibration. Chemical bonds vibrate at specific, characteristic frequencies when exposed to infrared radiation. Absorption of this radiation occurs only when two conditions are met:

- (i) The frequency of the infrared light matches the natural vibrational frequency of the molecular bond.
- (ii) The vibration causes a net change in the dipole moment of the molecule.

The instrument itself uses an interferometer to simultaneously measure all infrared frequencies. The resulting interference pattern (interferogram) is then mathematically transformed using a Fourier Transform algorithm into a conventional spectrum plotting absorbance versus wavenumber (cm^{-1}), which serves as a unique fingerprint of the material's chemical structure¹³⁶.

In my research work, FT-IR was utilized to characterize the electrode surface post-modification, ensuring the successful application of the conductive layer crucial for our sensing platform.

1.8.11 Scanning Electron Microscopy (SEM)

SEM functions by scanning a focused beam of high-energy electrons across the surface of a solid sample. The principle relies on the detection of signals generated from the interactions between the primary electron beam and atoms within the sample. The two most critical signals for imaging are:

- (i) Secondary electrons (SE): Low-energy electrons ejected from the sample surface. The yield of these electrons is highly sensitive to surface topography, providing a three-dimensional appearance and fine detail.
- (ii) Backscattered electrons (BSE): High-energy primary electrons that are elastically scattered back from the sample. The intensity of BSE is proportional to the atomic number of the elements in the sample, providing contrast based on elemental composition.

By mapping the intensity of either signal as the beam raster-scans the surface, a high-resolution image of the sample's morphology and composition is constructed¹³⁷.

In this work, SEM was utilized to characterize the electrode surface post-modification, ensuring the successful application of the conductive layer crucial for our sensing platform.

1.8.12 Overview of our research work

Table1.3: Details of the research activities

Experimental area		Successful outcome	
Solubility checked in	Water Ethanol Acetic Acid Methanol Acetate Buffer (pH) Phosphate Buffer (pH)	Soluble in	Ethanol Methanol Acetate Buffer (pH) Phosphate Buffer (pH 6.8)
Supporting electrolyte used	KCl Acetate Buffer (pH) Phosphate Buffer (pH)	Supporting electrolyte found effective	Phosphate Buffer (pH 6.8)
pH range checked	4 to 8	pH range used	5 to 7.4
Working electrode used	Gold Bare GCE Modified Electrode (Leucine, Alanine, PGA)	Working electrode used	Modified Electrode (PGA Modified GCE)

CHAPTER II

EXPERIMENTAL

CHAPTER II: EXPERIMENTAL

2.1 General

2.1.1 Electrochemical determination of antibiotics

Antibiotics are crucial in treating bacterial infections, but their misuse and overuse have led to the rise of antibiotic-resistant bacteria, posing a significant threat to global health^{50, 138}. Detecting antibiotics in various matrices, such as biological fluids, food and environmental samples is essential for monitoring their presence and mitigating their impact¹³⁹⁻¹⁴⁰. Electrochemical methods have emerged as promising techniques for the sensitive and selective determination of antibiotics due to their simplicity, cost-effectiveness, and high sensitivity³⁸.

Principles of electrochemical detection

Electrochemical detection involves measuring the current response resulting from the redox reactions of antibiotics at a suitable electrode surface. The technique relies on the application of a potential to the electrode and monitoring the resulting current, which is proportional to the concentration of the respective antibiotic. Various electrochemical techniques, such as cyclic voltammetry (CV), linear sweep voltammetry (LSV), differential pulse voltammetry (DPV), and square wave voltammetry (SWV), are commonly used for antibiotic detection^{38, 122, 141}.

Advantages of electrochemical methods

Electrochemical methods offer several advantages over traditional techniques, such as liquid chromatography-mass spectrometry (LC-MS) and immunoassays¹⁴¹. These advantages include:

- (i) High sensitivity: Electrochemical sensors can detect antibiotics at very low concentrations, making them suitable for trace analysis.
- (ii) Rapid response: Electrochemical detection provides quick results, allowing for real-time monitoring.
- (iii) Portability: Electrochemical sensors can be miniaturized and integrated into portable devices, enabling on-site analysis.
- (iv) Cost-effectiveness: Electrochemical methods are generally less expensive than other analytical techniques.

Recent advancements in electrochemical sensors

Recent advancements in electrochemical sensors have focused on enhancing their sensitivity

and selectivity through the use of nanomaterials and novel electrode modifications¹⁴¹. Some notable developments include:

- (i) Nanomaterials¹⁴¹: The incorporation of nanomaterials, such as graphene, metal nanoparticles, and metal-organic frameworks (MOFs), has significantly improved the performance of electrochemical sensors.
- (ii) Molecularly imprinted polymers (MIPs)¹⁴¹⁻¹⁴²: MIPs have been used to create selective recognition sites for specific antibiotics, enhancing the sensor's selectivity.
- (iii) Biosensors^{141, 124}: Electrochemical biosensors, which combine biological recognition elements with electrochemical detection, have shown great potential for antibiotic detection.

Roles: Electrochemical determination of antibiotics offers a powerful and versatile approaches for detecting these critical compounds in various matrices. With ongoing advancements in sensor technology, electrochemical methods are poised to play a significant role in addressing the challenges posed by antibiotic resistance and ensuring public health safety¹²⁴.

2.1.2 Electrochemical determination of antibiotic resistance

Antibiotic resistance is a significant and growing global health threat. It occurs when bacteria evolve mechanisms to resist the effects of antibiotics, rendering common treatments ineffective and leading to persistent infections. Detecting antibiotic resistance rapidly and accurately is crucial for effective treatment and management of infections. Electrochemical methods have emerged as powerful tools for the detection and analysis of antibiotic resistance due to their sensitivity, specificity, and rapid response.

Basics of electrochemical detection

Electrochemical detection methods involve monitoring the electrochemical behavior of bacterial cells or genetic markers of resistance at an electrode surface. These methods rely on the measurement of electrical signals, such as current generated by the interactions between the target molecules and the electrode or the materials modified on to the electrode surface. Common techniques include cyclic voltammetry (CV), differential pulse voltammetry (DPV), and electrochemical impedance spectroscopy (EIS), each providing unique insights into the resistance mechanisms from the measurement of either current or the parameters related to the antibiotic resistant.

Benefits of electrochemical techniques

The electrochemical determination of antibiotic resistance offers several advantages over traditional methods, such as culture-based techniques and molecular assays same as to the detection of antibiotics:

- (i) **High Sensitivity**^{38, 143, 121}: Electrochemical sensors can detect low levels of resistant bacteria or genetic markers.
- (ii) **Rapid Results**^{143, 121}: These methods provide quick detection, enabling timely decision-making for treatment.
- (iii) **Portability**¹⁴³: Electrochemical sensors can be miniaturized for on-site testing, making them suitable for point-of-care diagnostics.
- (iv) **Cost-Effectiveness**^{143, 38}: Compared to other analytical techniques, electrochemical methods are generally more affordable and require simpler instrumentation.

Recent advancement in electrochemical sensors for antibiotic resistance

Recent advancements in electrochemical sensors have enhanced their capability to detect antibiotic resistance:

Nanocomposites¹⁴⁴: Electrochemical sensors have emerged as transformative tools in the detection of antibiotic resistance. These innovative sensors can pinpoint antibiotic-resistant genes, observe alterations in bacterial cell membranes, and identify redox molecules linked to resistance. Their ability to deliver swift, highly sensitive, and affordable results makes them exceptionally suitable for real-time monitoring in both clinical and environmental contexts. The development of nanomaterials, such as carbon nanocomposites, has further elevated the performance of these sensors. By enhancing their sensitivity and selectivity, these advancements play a crucial role in addressing the global challenge posed by antimicrobial resistance.

Biosensors: Electrochemical biosensors combine biological recognition elements, such as enzymes, antibodies, or nucleic acids, with electrochemical detection to specifically identify resistant bacteria or resistance genes^{38, 122, 144}.

Applications of electrochemical detection

- (i) Electrochemical detection of antibiotic resistance has diverse applications:
- (ii) **Clinical Diagnostics**⁶: Rapid identification of resistant bacterial strains in clinical samples to guide appropriate antibiotic therapy.

- (iii) Environmental Monitoring¹²¹: Detection of antibiotic-resistant bacteria in water, soil, and wastewater to assess environmental contamination¹⁴⁵.
- (iv) Food Safety¹²¹: Monitoring antibiotic resistance in food products to ensure public health safety¹⁴⁶⁻¹⁴⁷.
- (v) Pharmaceutical Research¹²¹: Evaluating the efficacy of new antibiotics and resistance mechanisms.

Opportunities

Electrochemical determination of antibiotic resistance is a promising approach for addressing the challenges posed by antibiotic-resistant bacteria. With continued advancements in sensor technology and miniaturization, electrochemical methods are poised to become essential tools in clinical diagnostics, environmental monitoring, and public health³⁸.

If any bacteria are susceptible to any antibiotics, it acts on it either to kill the bacteria or to inhibit the growth of the bacteria. On the other hand, if any bacteria are resistant to any specific antibiotics, it may survive and continue to grow due to one or more resistance mechanisms.

The core objective of my research is to develop a rapid, sensitive, and cost-effective electrochemical testing technique which can facilitate early diagnosis of antibiotic resistance. Specifically, those antibiotics which mechanism of action indicates the inactivation or breakdown of antibiotic molecule upon interaction with that bacterium. Considering the mechanism of action of antibiotics, antibiotic molecules are selected from the cephalosporin group and quinolone group.

Cephalosporin antibiotics are having β -lactam ring and the antibiotics work by inhibiting or disrupting cell wall synthesis. The mechanism is, antibiotic molecule binds to penicillin-binding proteins (PBPs) in bacterial cell walls which results bacterial death. On the other hand, resistant bacteria have the capability to degrade antibiotic molecules by generating β -Lactamase enzyme¹⁴⁸. So, the quantitative determination of antibiotics over a period of time, after taking or administering antibiotics can give an idea about the nature of bacteria whether the bacteria are resistant or sensitive. Because, if any bacteria are sensitive against a specific antibiotic, then, the bacteria will be killed by the antibiotic. A very small portion of antibiotic from administered quantity will be degraded for the killing of bacteria and the remaining quantity of antibiotic can be determined. If the bacteria are resistant then, the concentration or peak response will decay gradually with respect to the time and continuous growth of

bacteria. So, β -lactamase enzyme was taken into consideration to conduct these studies with cephalosporin antibiotics.

Similarly, selected another molecule from quinolone group which is effective against both gram-positive and gram-negative bacteria. The mechanism is also different. It works by inhibiting bacterial DNA gyrase and topoisomerase IV, which are essential for DNA replication and transcription. Quinolones passively diffuse through porins in the gram-negative outer membrane and once inside, it accumulates due to interactions with cytoplasmic components. A suitable gram-negative bacteria strains *E. coli* ATCC 8739 was selected to conduct this study.

This determination technique aims to provide real-time detection of resistant bacterial strains in biological samples like blood or urine. The detection would be through the identification of specific resistance markers. The technique will leverage the high sensitivity and specificity of electrochemical sensors, with the goal of improving patient outcomes by facilitating timely and accurate treatment decisions.

2.2 Chemicals, reagents, reference strains and biological enzyme used

Several chemicals, reagents and active pharmaceuticals ingredients (API; antibiotic molecules) used in different types of experiment required to conduct the research-

Common chemicals and reagents used:

- (i) Deionized water (Department of Chemistry, Dhaka University)
- (ii) Potassium chloride (Merck, Germany) (Expiry: February 2026)
- (iii) Potassium bromide (Merck, Germany) (Expiry: May 2027)
- (iv) Potassium ferrocyanide (Merck, Germany) (Expiry: June 2027)
- (v) Nitrogen (99.997% dry) (BOC, Bangladesh)-Used for N₂ purging
- (vi) Potassium phosphate (Monobasic) (Merck, Germany) (Expiry: January 2027)
- (vii) Sodium hydroxide pellets (A.R. grade) (Scharlau) (Expiry: November 2028)
- (viii) Potassium chloride (Merck, Germany) (Expiry: June 2026)
- (ix) Sodium acetate (Merck, Germany) (Expiry: March 2026)
- (x) Acetic acid (Sigma-Aldrich, GmbH) (Expiry: July 2025)
- (xi) Methanol (Merck, Germany) (Expiry: December 2026)
- (xii) Ethanol (Absolute for analysis) (Merck, Germany) (Expiry: March 2026)

Details of *E.coli* ATCC reference strain of living microorganism

Name: *E.coli* ATCC

Reference number: ATCC 8739

Catalog number: 0483

Lot number: 483-1416

Release date: 13.05.2024

Expiration date: 30.04.2026

100 μ L = 10 to 100 cfu

Source: Square Pharmaceuticals PLC.

Details of β -lactamase (biological enzyme)

Product name: LACTAMATOR (Sterile liquid)

Product code: # LCT-LQS100

Batch: 019.LQS100.00124

Manufacturing date: January 2024

Expiry date: January 2027

Release test result:

Cephalosporinase activity: Specification: >100 IU/ml, Test Result: 186 IU/ml

Penicillinase activity: Specification: >1000 IU/ml, Test Result: 2402 IU/ml

Source: Square Pharmaceuticals PLC.

Antibiotics used:

- (i) Ceftibuten Dihydrate INN (Square Pharmaceuticals PLC.) (Batch No.- 1000088413; Mfg. Date: Jun'22; Expiry Date: May'27)
- (ii) Cefuroxime Sodium BP (Square Pharmaceuticals PLC.) (Batch No.- 1000077568; Mfg. Date: Mar'21; Expiry Date: Feb'26)
- (iii) Levofloxacin Hemihydrate USP (Square Pharmaceuticals PLC.) (Batch No.- 1000083494; Mfg. Date: Jun'21; Expiry Date: May'26)

2.3 Instruments used

The research study carried out using following instrument-

- (i) Electrochemical Workstation: Two electrochemical workstations were used to conduct the research. Zahner Zaehnum Pro developed by Zahner-Elektrik GmbH & Co., Kronach, Germany and CH Electrochemical workstation.

- (ii) Electrochemical Cell: A pyrex glass micro undivided cell with custom build Teflon cap.
- (iii) Working Electrode: Glassy Carbon Electrode (GCE) with a diameter of 3 mm was used as working electrode.
- (iv) Reference Electrode: Ag(s)/AgCl(s) electrode immersed in saturated solution of KCl was used as reference electrode.
- (v) Counter Electrode: Pt electrode/ wire was used as counter electrode.
- (vi) An AGE (Velp Scientifica) magnetic stirrer with a Teflon coated magnetic bar is always immersed in the cell to ensure homogeneous mixing of the solution.
- (vii) A pH meter (Orion 2 Star made by Thermo Electron Corporation).
- (viii) Four-digit and Five-digit weighing balances were used to measure the weight of the respective reagents and chemicals.
- (ix) Fourier transform infrared (FT-IR) spectroscopy, Perkin Elmer, Model- Spectrum Two, at Square Pharmaceuticals PLC.
- (x) Field Emission Scanning electron microscopy (FESEM), Model- Sigma 300 by Carl Zeiss AG, Germany at Genome Centre, Jashore University of Science & Technology.
- (xi) Drying Oven, at Square Pharmaceuticals PLC.

2.4 Experimental setup and methodology

2.4.1 Potentiostat

The **Zahner Zennium Pro** operates by controlling the voltage and current between a working electrode and a reference electrode in an electrochemical cell. Simplified explanation of its mechanism is as below-

Electrochemical cell setup: The Zennium Pro is connected to an electrochemical cell, which typically includes a working electrode, a reference electrode, and a counter electrode.

Potential control: The potentiostat/galvanostat component of the Zennium Pro applies a controlled potential (voltage) to the working electrode relative to the reference electrode.

Current measurement: The device measures the resulting current that flows between the working and counter electrodes. This current is directly related to the electrochemical reactions occurring at the working electrode.

Frequency response analysis (FRA): For impedance measurements, the Zennium Pro applies an alternating current (AC) signal and measures the response of the system over a range of frequencies (up to 8 MHz). This helps in analyzing the electrochemical properties of the system.

Data acquisition and analysis: The measured current and impedance data are recorded and analyzed using the Zennium Pro's software, providing insights into the electrochemical processes. The system configures the experimental control parameters to apply a cyclic linear potential sweep to the working electrode, with the aim of generating the corresponding current–potential response. This electrochemical sweep is typically characterized by its initial potential (E_i), switching potential (E_s), final potential (E_f), and the scan rate expressed in volts per second (V/s). We used the below potentiostat for the measurement of impedance spectra of the respective electrodes.



Figure 2. 1: Computer controlled zahner zennium pro potentiostat

The **CH electrochemical workstation** (Instrument Model: CHI650E) operates by controlling the voltage and current between a working electrode and a reference electrode in an electrochemical cell. The simplified explanation of its mechanism:

Electrochemical cell setup: The workstation is connected to an electrochemical cell, which typically includes a working electrode, a reference electrode, and a counter electrode.

Potential control: The workstation applies a controlled potential (voltage) to the working electrode relative to the reference electrode.

Current measurement: The device measures the resulting current that flows between the working and counter electrodes. This current is directly related to the electrochemical reactions occurring at the working electrode.

Data acquisition and analysis: The measured current and potential data are recorded and analyzed using the workstation's software, providing insights into the electrochemical processes. The CH Electrochemical Workstation is designed for general-purpose electrochemical measurements and offers features such as high-speed data acquisition, multi-functional use, and a user-friendly interface. All the voltammetric runs were conducted by using above potentiostat.



Figure 2. 2: Computer controlled CH electrochemical workstation potentiostat

2.4.2 Electrochemical cell

This study employed a three-electrode electrochemical cell configuration, enclosed with a Teflon cap to maintain system integrity. The targeted electrochemical reaction occurs at the working electrode, where electron transfer generates a measurable current known as the faradaic current. To maintain electrochemical equilibrium, the counter electrode is regulated by a potentiostatic circuit, facilitating an opposing electron flow that compensates for the faradaic process occurring at the working electrode.

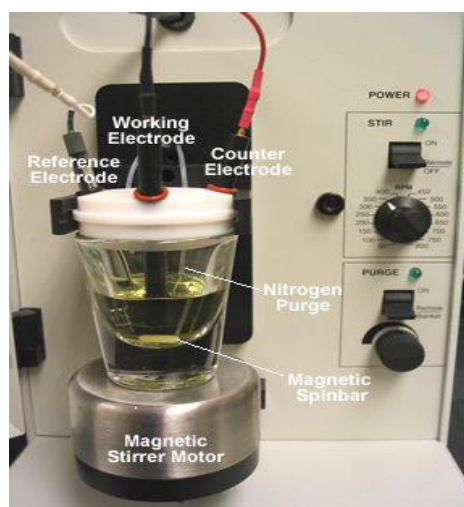


Figure 2. 3: Three-electrode cell set-up consisting of a working electrode, a reference electrode and a counter or auxiliary electrode with a C3 cell stand procured from BASi, US

2.4.3 Electrodes

- (i) Working electrode:
 - (a) Glassy carbon electrode (GCE) with 3 mm diameter glassy carbon disc
 - (b) Poly glutamic acid (PGA) modified glassy carbon electrode
- (ii) Reference electrode: Ag/AgCl electrode
- (iii) Counter electrode: Pt electrode/ Pt Wire

The working electrode serves as the site for redox transformations of the analyte, where electron transfer reactions are actively monitored. The reference electrode maintains a stable potential and facilitates the flow of a minimal current during electrolysis, ensuring consistent behavior at the working electrode. In a three-electrode configuration, the counter electrode typically composed of an inert metal completes the circuit and supports the current flow required to balance the electrochemical processes occurring at the working electrode.

The electrodes were cleaned and polished using alumina powder and nonconductive deionized water. The electrodes were cleaned frequently based on deposition on electrode surface.

2.4.4 Preparation of glassy carbon electrode

The working electrode used in this study is glassy carbon electrode (GCE) procured from the Bio- analytical system (BASi), USA. The electrode preparation includes the polishing and conditioning of the electrode. The glassy carbon electrode was polished with 0.5-micron fine alumina powder on a wet polishing cloth. To do so a part of the cloth was made wet with deionized water and alumina powder was sprinkled on it. The glassy carbon electrode was then polished on this surface by softly pressing the electrode against the polishing surface at the end for 5-10 minutes. The electrode was then thoroughly washed with deionized water and sonicated for 5 minutes in deionized water. Then wiped off the electrode with lint free tissue and at this point the electrode surface would look like a shiny black mirror.

2.4.5 Removing dissolved oxygen from the system

Before the experiment, it is necessary to remove the dissolved oxygen and was done by purging N₂ gas (99.99% pure and dry) through the solution. During each voltammetric run the flow of N₂-gas was maintained on the surface of the solution top to avoid the mixing of O₂-gas.

2.4.6 Preparation of poly glutamic acid (PGA) modified glassy carbon electrode

Modification of glassy carbon electrode (GCE) with polyglutamic acid (PGA) involves depositing a thin, adherent layer of PGA onto the electrode surface. A step-by-step procedure was followed for PGA modification, along with key the optimization of glassy carbon surface. To clean the surface of glassy carbon electrode, the surface of the electrode polished with 0.3 μ alumina and rinsed it with deionized water. Then the electrode was immersed in deionized water and sonicated for 30 s. The surface was wiped with lint free tissue and checked the surface visually to make sure that it was cleaned properly. 1 mg/mL of PGA solution in phosphate buffer with pH 6.8 was prepared and 10 mL of the solution was taken in the electrochemical cell. N₂ Purging was done to remove dissolved oxygen and ensure inert atmosphere. Then the cleaned glassy carbon electrode in PGA solution was immersed for electrodeposition. Electrochemical pretreatment of GCE was executed by applying potential +1.8 V for about 300 seconds. After that, CV was performed at potential window –1.5 V to +2.0 V with scan rate 100 mV/s for 10 to 15 cycles. Finally, activated modified electrode was checked by scanning a single CV within potential window –1.0 V to +2.0 V and with a scan rate 50 mV/s. The modified electrode was always rinsed prior to each electrochemical run.

2.4.7 Preparation of various stock solutions

Supporting electrolyte: 0.1 M KCl solution was prepared in a 100 mL volumetric flask by dissolving 0.747 g analytical grade KCl into deionized water.

Phosphate buffer: Prepared Phosphate Buffer by taking in a proportion of Monobasic Potassium Phosphate 1.36 g in 100 ml and Sodium Hydroxide to adjust pH (pH 4.5 to pH 7.4).

Ceftibuten solution: 10 ppm or 10 mg/1000 mL Ceftibuten stock solution was prepared in Phosphate Buffer by dissolving 20 mg of Ceftibuten in 100 mL of Phosphate Buffer and then further diluting 5 mL into 100 mL in phosphate buffer. The voltammogram was taken by injecting different amount of stock solution using micropipette into 10 mL raw Phosphate Buffer solution (with calculation 1 μ L turns into 1 ppb).

Cefuroxime solution: 10 ppm or 10 mg/1000 mL Cefuroxime stock solution was prepared in Phosphate Buffer by dissolving 20 mg of Ceftibuten in 100 mL of Phosphate Buffer and then further diluting 5 mL into 100 mL in phosphate buffer. The voltammogram was taken by injecting different amount of stock solution using micropipette into 10 mL raw Phosphate

Buffer solution (with calculation 1 μ L turns into 1 ppb).

Levofloxacin solution: 10 ppm or 10 mg/1000 mL Cefibuten stock solution was prepared in Phosphate Buffer by dissolving 20 mg of Levofloxacin in 100 mL of Phosphate Buffer and then further diluting 5 mL into 100 mL in phosphate buffer. The voltammogram was taken by injecting different amount of stock solution using micropipette into 10 mL raw Phosphate Buffer solution (with calculation 1 μ L turns into 1 ppb).

2.4.8 pH maintenance of solution

For the Measurement of pH of the prepared solutions, ORION 2 STAR pH measurement kit (Figure 2.4) was used.

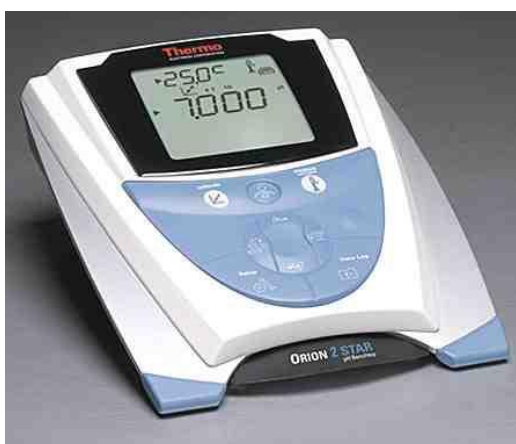


Figure 2. 4: The ORION 2 STAR pH measurement kit

2.4.9 Precautions

The following precautions must be taken to perform any electrochemical experiments:

- (i) The electrodes should not be connected until the reset program run was completed.
- (ii) The cell should never be disconnected while the experiment is in process.
- (iii) The computer should never be turned off during an experiment.
- (iv) Before turning off the main switch, all the electrodes should be disconnected from the system and the system should be shut down properly.

2.4.10 Cyclic voltammetry (CV)

Cyclic Voltammetry (CV) is a widely utilized electrochemical technique for investigating redox behavior at electrode interfaces. In this study, CV was selected to examine the electron transfer processes occurring at a polarizable electrode surface. The method provides valuable insights into the kinetics and mechanisms of redox reactions, as well as the adsorption characteristics of reactants and products on the electrode^{129, 132, 131}.

Key analytical parameters extracted from CV data include the cathodic peak current (i_{pc}), anodic peak current (i_{pa}), cathodic peak potential (E_{pc}), and anodic peak potential (E_{pa}). These variables, in conjunction with controlled scan rates, are instrumental in deciphering the reaction dynamics and electrochemical behavior of the system under investigation.

CV is regarded as one of the most versatile and informative electroanalytical techniques for probing redox systems. It allows for rapid modulation of the electrode potential to identify redox-active species. Once detected, these species can be characterized based on the peak potentials observed in the cyclic voltammogram and their response to different scan rates. Typically employed as an initial diagnostic tool in electrochemical studies, CV involves applying a time-dependent potential to an electrode immersed in a quiescent solution and recording the resulting current. The potential sweep, which may range from a few millivolts to several hundred millivolts per second, generates a characteristic profile known as a voltammogram depicting the relationship between applied potential and measured current during both anodic and cathodic cycles.

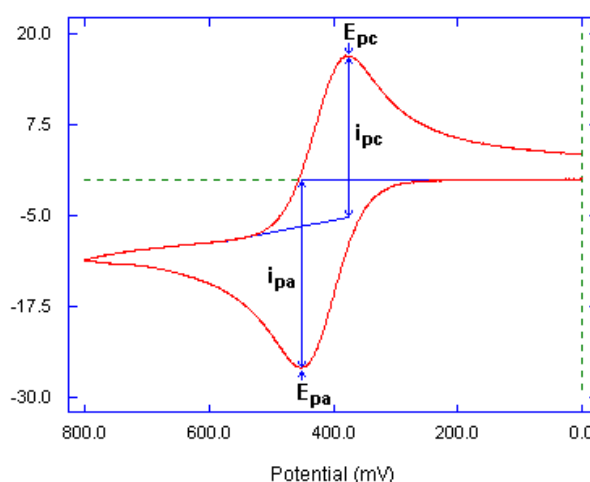


Figure 2. 5: A typical cyclic voltammogram and it's various parameters

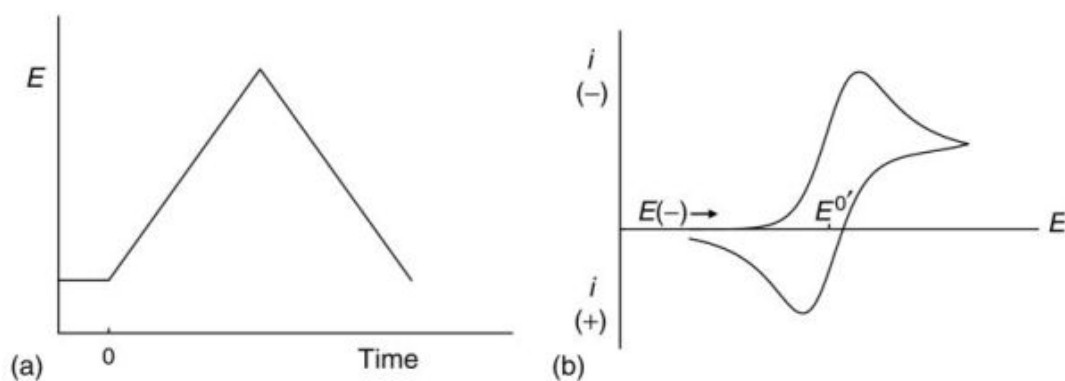


Figure 2. 6: A typical excitation signal in cyclic voltammetry (a) and corresponding voltammogram (b)

Under diffusion-controlled mass transfer conditions at a stationary electrode, cyclic voltammetry (CV) employs a symmetrical triangular potential waveform, typically scanned at rates ranging from 1 mV/s to several hundred millivolts per second. In this technique, the current response is recorded as a function of the scan rate, providing insight into the electrochemical behavior of the analyte.

The potential of the working electrode is precisely regulated relative to a reference electrode, commonly Ag/AgCl. During the experiment, the applied potential is linearly swept toward a more negative value and subsequently reversed back to the initial potential. This bidirectional scan generates a characteristic current peak during the forward sweep when the analyte undergoes reduction within the scanned potential window. As the potential approaches the reduction threshold of the analyte, the current increases, reflecting the onset of the redox process.

Cyclic voltammetry (CV) serves as a powerful diagnostic tool for elucidating various electrochemical properties and reaction mechanisms. Specifically, this technique provides insights into:

- (i) The reversibility of electrochemical reactions, offering a rapid and efficient approach for analyzing diverse systems
- (ii) The direct detection of transient reactive intermediates, enabling mechanistic exploration
- (iii) The characterization of sequential electrochemical and chemical steps within complex reaction pathways
- (iv) The redox behavior of electron donor acceptor pairs, including identification of oxidation and reduction potentials
- (v) The evaluation of charge transfer processes at the electrode solution interface, along with the determination of the associated rate constants

An electrochemical system containing species 'O' capable of being reversibly reduced to 'R' at the electrode is given by,



Nernst equation for the system is (2)

$$E = E^o + \frac{0.059}{n} \log \frac{C_0^S}{C_R^S}$$

Where, E = Potential applied to the electrode

E^0 = Standard reduction potential of the couple versus reference electrode

n = Number of electrons in Eq. I

C_0^S = Surface concentration of species 'O'

C_0^S = Surface concentration of species 'R'

A redox couple that changes electrons rapidly with the working electrode is termed as electrochemically reverse couple. For Eq. II, the relation gives the peak current i_{pc} ,

$$i_{pc} = 0.4463 \text{ nFA } (D\alpha)^{1/2} C \quad (3)$$

$$\alpha = \left(\frac{nFv}{RT} \right) = \left(\frac{nv}{0.026} \right)$$

where, i_{pc} = peak current in amperes

F = Faraday's constant (approximately 96500)

A = Area of the working electrode in cm^2

V = Scan rate in volt/sec

C = Concentration of the bulk species in mol/L

D = Diffusion coefficient in cm^2/se

In terms of adjustable parameters, the peak current is given by the Randless-Sevcik equation,

$$i_{pc} = 2.69 \times 10^5 n^{3/2} A D^{1/2} C v^{1/2} \quad (4)$$

The peak potential E_p for reversible process is related to the half wave potential $E_{1/2}$, by the expression,

$$E_{pc} = E_{1/2} - 1.11 \left(\frac{RT}{nF} \right) \quad \text{at } 25^\circ\text{C} \quad (5)$$

$$E_{pc} = E_{1/2} - \left(\frac{0.0285}{n} \right) \quad (6)$$

The relation relates the half wave potential to the standard electrode potential

$$E_{1/2} = E^0 - \frac{RT}{nF} \ln \frac{f_{red}}{f_{ox}} \left(\frac{D_{ox}}{D_{red}} \right)^{1/2}$$
$$E_{1/2} = E^0 - \frac{RT}{nF} \ln \left(\frac{D_{ox}}{D_{red}} \right)^{1/2} \quad (7)$$

Assuming that the activity coefficient f_{ox} and f_{red} are equal for the oxidized and reduced species involved in the electrochemical reaction.

From equation (VI), we have,

$$E_{pa} - E_{pc} = 2.22 \left(\frac{RT}{nF} \right) \quad \text{at } 25^\circ\text{C} \quad (8)$$

$$\text{or, } E_{pa} - E_{pc} = \frac{0.059}{n} \quad \text{at } 25^\circ\text{C} \quad (9)$$

This is a good criterion for the reversibility of electrode process. The value of i_{pa} and i_{pc} should be close for a simple reversible couple,

$$i_{pa}/i_{pc} \approx 1 \quad (10)$$

And such a system $E_{1/2}$ can be given by,

$$E_{1/2} = \left(\frac{E_{pa} + E_{pc}}{2} \right)$$

Irreversible and quasi-reversible systems

For irreversible processes (those with sluggish electron exchange), the individual peaks are reduced in size and widely separated. Totally irreversible systems are characterized by a shift of the peak potential with the scan rate:

$$E_p = E^\circ - (RT/\alpha n_a F)[0.78 - \ln(k^\circ/(D)^{1/2}) + \ln(n_a F \alpha / RT)^{1/2}]$$

where α is the transfer coefficient and n_a is the number of electrons involved in the charge-transfer step. Thus, E_p occurs at potentials higher than E° , with the overpotential related to k° (standard rate constant) and α . Independent of the value k° , such peak displacement can be compensated by an appropriate change of the scan rate. The peak potential and the half-peak potential (at 25°C) will differ by $48/\alpha n$ mV. Hence, the voltammogram becomes more drawn-out as αn decreases.

The peak current, given by:

$$i_p = (2.99 \times 10^5) n(\alpha n_a)^{1/2} A C D^{1/2} \nu^{1/2}$$

is still proportional to the bulk concentration, but will be lower in height (depending upon the value of α). Assuming $\alpha = 0.5$, the ratio of the reversible-to-irreversible current peaks is 1.27 (*i.e.* the peak current for the irreversible process is about 80% of the peak for a reversible one). For quasi-reversible systems (with $10^{-1} > k^\circ > 10^{-5}$ cm/s) the current is controlled by both the charge transfer and mass transport. The shape of the cyclic voltammogram is a function of the ratio $k^\circ/(\pi \nu n F D / RT)^{1/2}$. As the ratio increases, the process approaches the reversible case. For small values of it, the system exhibits an irreversible behavior. Overall, the voltammograms of a quasi-reversible system are more drawn out and exhibit a larger

separation in peak potentials compared to a reversible system. Unlike the reversible process in which the current is purely mass transport controlled, currents due to quasi-reversible process are controlled by a mixture of mass transport and charge transfer kinetics. The process occurs when the relative rate of electron transfer with respect to that of mass transport is insufficient to maintain Nernst equilibrium at the electrode surface¹³¹.

Calculating the Heterogeneous Charge Transfer Rate constant:

Calculation of k_f (reversible system)

Working formula $i_{pc} = nFAC_0 k_f$

Input parameters

i_{pc} = cathodic peak current, A

n = no. of electron involved in the reaction

F = coulomb / mole

A = area of the electrode, cm^2

C_0 = concentration, mol/L

Calculation of k_f (irreversible system):

Working formula $E_{pc} = E_{1/2} - b [0.52 - \frac{1}{2} \log (b/D) - \log k_f + \frac{1}{2} \log v]$

Input parameters:

E_{pc} = cathodic peak potential, V

b = peak potential in terms of Tafel slope

Tafel slope = $2.303RT / (\alpha n_a F)$

F = coulomb / mol

αn_a is obtained using the relation

$$(E_{pc})_2 - (E_{pc})_1 = RT / (\alpha n_a F) \ln \sqrt{(v_1 / v_2)}$$

$(E_{pc})_1$ = cathodic peak potential (V) at scan rate v_1

$(E_{pc})_2$ = cathodic peak potential (V) at scan rate v_2

T = temperature at K

R = gas constant, joules /mol/degree

$E_{1/2} = -0.25753 + 1.857 RT / \alpha n_a F$ (value of from the above calculation)

D = diffusion coefficient is calculated using the relation

$$i_{pc} = 2.99 \times 10^5 n (\alpha / n_a)^{1/2} A D^{1/2} C v^{1/2}$$

i_{pc} = cathodic peak current, A

n = no. of electron involved in the reaction

A = area of the electrode, cm^2

C_0 = concentration, mol/L

v =scan rate

2.4.11 Differential pulse voltammetry (DPV)

Differential pulse voltammetry (DPV) is a sensitive electroanalytical technique employed for precise electrochemical measurements. It is conceptually derived from linear sweep or staircase voltammetry, wherein a sequence of uniform voltage pulses is superimposed onto a gradually increasing baseline potential. The current is sampled immediately before each potential increment, and the difference in current is plotted against the applied potential. This sampling strategy effectively minimizes the influence of capacitive (charging) currents, thereby enhancing analytical resolution^{149, 133}.

In contrast, normal pulse voltammetry (NPV) involves applying progressively larger potential pulses and comparing the resulting current with that measured at a fixed baseline potential. Another variant, square wave voltammetry (SWV), can be viewed as a specialized form of DPV, where equal durations are allocated to both the baseline and pulse potentials within each cycle.

The waveform used in DPV or differential pulse polarography (DPV/P) typically consists of small, constant-amplitude pulses superimposed on a staircase potential profile (refer to Figure 2.7). Unlike NPV, DPV samples the current twice during each pulse cycle—once immediately before the pulse and again at the end of the pulse and the difference between these two readings is recorded and analyzed¹⁴⁹.

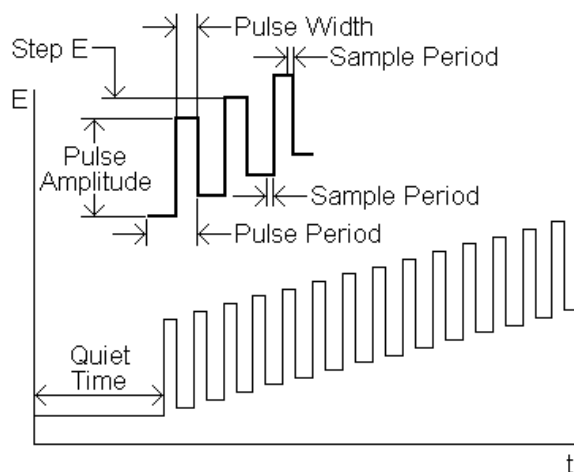


Figure 2. 7: Excitation signal and Potential wave form for differential pulse voltammetry

Differential pulse voltammetry has these characteristics:

- (i) Reversible reactions show symmetrical peaks, and irreversible reactions show asymmetrical peaks.
- (ii) The peak potential is equal to $E_{1/2}^{\text{r}} - \Delta E$ in reversible reactions, and the peak current is proportional to the concentration.
- (iii) The detection limit is about 10^{-8} M.

2.4.12 Electrochemical impedance spectroscopy (EIS)

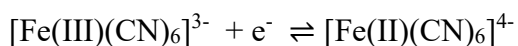
Electrochemical Impedance Spectroscopy (EIS) is a powerful technique used to study the electrical properties of electrochemical systems. The EIS experiment begins with the setup of an electrochemical cell, which includes a working electrode, a reference electrode, and a counter electrode. A small amplitude sinusoidal voltage or current signal is applied to the working electrode. This signal typically ranges from a few millihertz (mHz) to several megahertz (MHz). The response of the electrochemical system to the applied AC signal is measured. This response includes both the amplitude and phase shift of the current relative to the applied voltage. The frequency of the applied signal is varied over a wide range, often spanning several decades (e.g., from 0.01 Hz to 100 kHz). This allows the characterization of different processes occurring within the electrochemical system. The measured impedance data (complex impedance) is analyzed using equivalent electrical circuits. These circuits model the electrochemical processes, such as charge transfer resistance, double-layer capacitance, and diffusion impedance^{135, 150-151}.

Nyquist and Bode Plots: The impedance data is often represented in the form of Nyquist plots (real vs. imaginary impedance) and Bode plots (magnitude and phase vs. frequency). These plots help in identifying and interpreting the electrochemical processes.

Electrochemical Sensing: EIS is used to develop and optimize electrochemical sensors for detecting various analytes^{135, 150}.

2.4.13 Standardization of the system and system suitability check

The system suitability was verified through the electrochemical set-up and run using a standard Potassium Ferrocyanide (in aqueous 0.1M KCl solution). In this experiment we have studied the following redox couple at a glassy carbon electrode-



The reaction above was studied electrochemically by pumping electrons into the system from a GCE electrode and by measuring the change in the flow of current during the reaction. The following are the cyclic voltammograms of the solution (potassium ferrocyanide in aqueous 0.1M KCl solution) were obtained at different scan rate.

Experimental procedure for cyclic voltammetry

The electrochemical cell was assembled and subsequently filled with 10 mL of a supporting electrolyte solution comprising KCl, acetate buffer, and phosphate buffer. Prior to measurement, the solution was de-aerated for 5 minutes to eliminate dissolved oxygen, followed by a 10-second stabilization period to ensure quiescence. To establish the potential window, an initial scan was performed using only the supporting electrolyte, yielding the background voltammogram. Subsequent voltammetric measurements were conducted with the analyte dissolved in the same electrolyte system under two distinct experimental conditions: (i) different scan rates and (ii) different analyte concentrations.

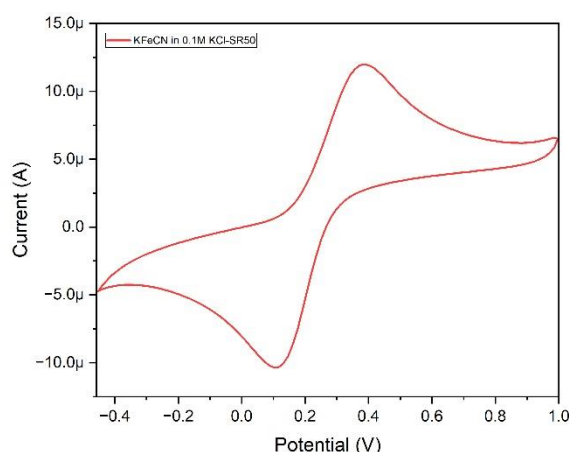


Figure 2. 8: Voltammogram of potassium ferrocyanide in 0.1M KCl at scan rate of 50 mV/s

Table 2.1: Current-potential data Fe(III)/Fe(II) in potassium ferrocyanide solution in 0.1 M KCl at a platinum electrode at different scan rate

Scan rate, $\nu\text{V/s}$	Cathodic peak potential, E_{pc}/mV	Anodic peak potential, E_{pa}/mV	Cathodic peak current, $-i_{pc}/\mu\text{A}$	Anodic peak current, $i_{pa}/\mu\text{A}$
0.020	101	386	7.25	8.39
0.040	101	386	9.32	10.79
0.060	101	386	11.39	13.19
0.080	101	386	13.46	15.58
0.100	101	386	15.53	17.98

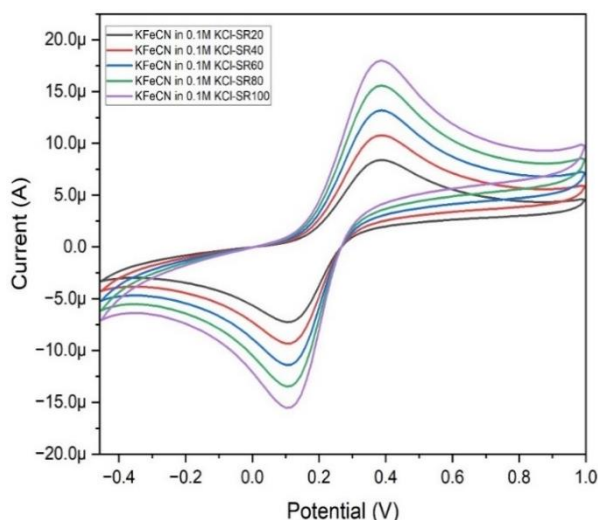


Figure 2. 9: CV of 1 mM potassium ferrocyanide in 0.1M KCl solution at different scan rates

Table 2.2: Calculated cyclic voltammograms data Fe(III)/Fe(II) in potassium ferrocyanide solution in 0.1 M KCl at a platinum electrode at different scan rate

Scan rate, ν , V/s	$\sqrt{\nu}$, V/s	Peak separation, $\Delta E = (E_{pc} - E_{pa})/\text{mV}$	Peak current ratio, i_{pc}/i_{pa}	$i_{pc}/\sqrt{\nu}$
0.020	0.141	285	0.86	51.27
0.040	0.200	285	0.86	46.60
0.060	0.245	285	0.86	46.50
0.080	0.283	285	0.86	47.59
0.100	0.316	285	0.86	49.11

From the current voltage data, the peak current ratio found unity and the peak separation value is 58 mV which are the major criteria for reversible process for one electrode system. For reversible process, peak current (i_p) represent according to the Randless-Sevcik equation as follow:

$$i_p = (2.69 \times 10^5) n^{3/2} A D^{1/2} C \nu^{1/2}$$

Taking the electrode area, $A = 0.025 \text{ cm}^2$ and $n = 1$ [for the Fe(II)/Fe(III) redox couple] and $C = 1 \times 10^{-3} \text{ M}$ ($1 \times 10^{-6} \text{ mol/cm}^3$), i_p = peak current in ampere, the diffusion co-efficient (D) was calculated from the slope of the plot i_{pa} vs $\nu^{1/2}$. The diffusion co-efficient (D) for the Fe(II)/Fe(III) redox couple was calculated and found very close to the literature value.

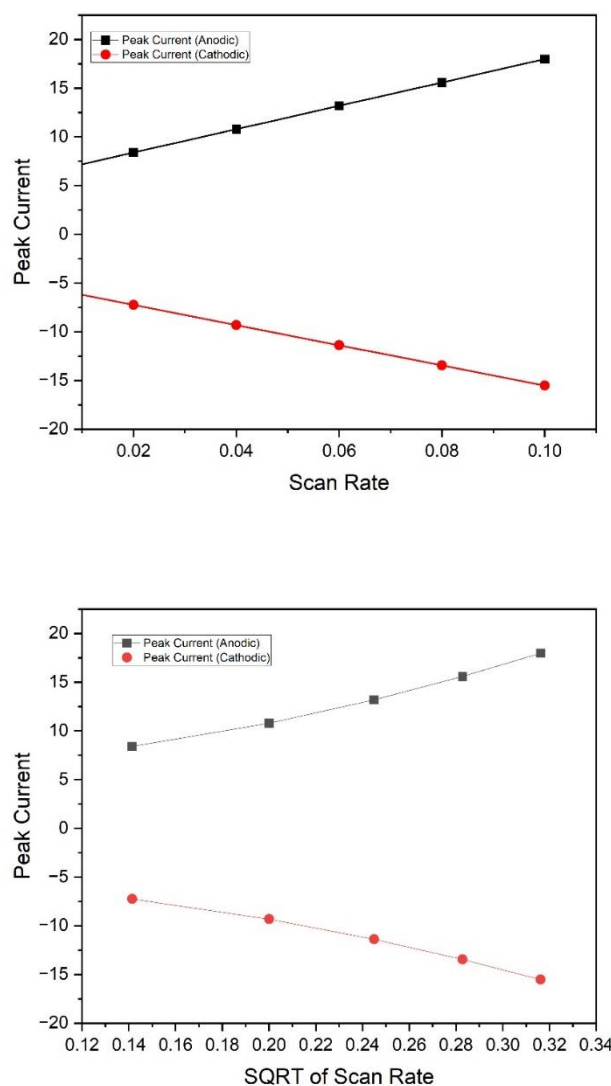


Figure 2. 10: Change of peak current with the square root of scan rate. Upper is the anodic and the lower is cathodic peak currents

The electrochemical reversibility, the voltammetric parameters such as, peak potential, peak separation and the peak current varying current function for the above ferri-ferro couple satisfy the theoretical values. Therefore, we use our present electrochemical set-up without further modification.

The above CV experiment was performed, which allowed to optimize the whole system. The diffusion and the kinetic parameters calculated was perfectly match with the literature values. So, we can say that the present experimental set-up was optimized.

2.4.14 Fourier transform infrared (FTIR) spectroscopy

Fourier-Transform Infrared (FTIR) spectroscopy is a powerful technique for analyzing molecular structure by measuring the absorption of infrared radiation corresponding to vibrational transitions in chemical bonds. The core innovation over dispersive IR is the use of an interferometer, typically a Michelson interferometer, to encode spectral information¹³⁶. The familiar frequency-domain spectrum ($B(\bar{\nu})$), absorbance versus wavenumber (cm^{-1}), is obtained by applying a Fourier Transform to the interferogram:

$$B(\bar{\nu}) = \int I(\delta) \cos(2\pi\bar{\nu}\delta) d\delta \quad (1)$$



Figure 2. 11: Fourier transform infrared (FT-IR) spectroscopy, Perkin Elmer

Sample Preparation: The chosen method must be appropriate. KBr pellets require completely dry reagents and fine grinding to minimize light scattering. For Attenuated Total Reflectance (ATR), ensuring firm, uniform contact between the sample and the crystal is paramount to obtain a valid spectrum. Note that ATR spectra exhibit wavelength-dependent intensity differences compared to transmission spectra.

Precautions: The instrument must be purged with dry, CO_2 -free air or nitrogen to eliminate spectral artefacts from atmospheric water vapour and CO_2 . Background scans must be collected immediately prior to sample analysis under identical conditions (resolution, purge state, scan number).

2.4.15 Field emission scanning electron microscopy (FESEM)

Field emission scanning electron microscopy (FESEM) is an advanced electron microscopy technique used for high-resolution imaging of solid material surfaces. Its operation is based

on interrogating a sample with a focused beam of high-energy electrons in a raster pattern and detecting the various signals emitted from the sample's surface due to electron-matter interactions.



Figure 2. 12: Field Emission Scanning Electron Microscopy (FESEM), Model- Sigma 300
by Carl Zeiss AG, Germany

Sample Preparation: Need to make sure the samples are clean, dry, and securely mounted on an appropriate stub using conductive adhesive (e.g., carbon tape) to ensure electrical grounding.

CHAPTER III

RESULTS AND DISCUSSION

CHAPTER III: RESULTS AND DISCUSSION

This chapter contains four sections. In the first section (Section 3.1), a detailed of the modification process of glassy carbon electrode using *L*-glutamic acid and the characterization of possible *L*-glutamic acid polymer layer formation was described. In the second, third and fourth sections (Section 3.2, 3.3 and 3.4), the electrochemical study details of the antibiotics ceftibuten, cefuroxime and levofloxacin, respectively as well as the detection of possible antibiotic resistance techniques are described.

Electrochemical techniques, such as cyclic voltammetry (CV), linear sweep voltammetry (LSV) and differential pulse voltammetry (DPV) were used for the quantitative determination of the selected antibiotics as well as the indication of antibiotic resistance.

Poly-glutamic acid (PGA) modified glassy carbon electrode was used as working electrode. The reason behind using modified electrode is to get better response and reproducible data rather by using bare glassy carbon electrode or activated glassy carbon.

Antibiotic molecules ceftibuten and cefuroxime were selected from the cephalosporin group. Both antibiotics are having β -lactam ring. These antibiotics work by inhibiting or disrupting cell wall synthesis. The mechanism is, antibiotic molecule binds to penicillin-binding proteins (PBPs) in bacterial cell walls which results bacterial death. In this mechanism, a certain quantity of antibiotic is degraded to kill the bacteria and the remaining quantity of antibiotic is excreted from the body. On the other hand, resistant bacteria have the capability to degrade antibiotic molecules by generating β -Lactamase enzyme. So, the quantitative determination of antibiotics over a period of time, after taking or administering antibiotics can give an idea about the nature of bacteria whether the bacteria are resistant or sensitive. Because, if any bacteria are sensitive against a specific antibiotic, then, the bacteria will be killed by the antibiotic. A very small portion of antibiotic from administered quantity will be degraded for the killing of bacteria and the remaining quantity of antibiotic can be determined. If the bacteria are resistant then, the concentration or peak response will decay gradually with respect to the time and continuous growth of bacteria. β -lactamase enzyme was taken into consideration to conduct these studies with ceftibuten and cefuroxime.

On the other hand, to see the suitability of electrochemical methods on detection of antibiotic resistance other than β -lactams, selected another molecule levofloxacin from quinolone group which is effective against both gram-positive and gram-negative bacteria. Its mode of action diverges from conventional pathways, targeting bacterial DNA gyrase and topoisomerase IV enzymes that play a crucial role in facilitating DNA replication and transcription.

Levofloxacin passively diffuses through porins in the Gram-negative outer membrane and once inside, it accumulates due to interactions with cytoplasmic components. A suitable gram-negative bacteria strains *E. coli* ATCC 8739 was selected to conduct this study.

3.1 Modification of working electrode with poly glutamic acid (PGA)

Modification of glassy carbon electrode (GCE) with polyglutamic acid (PGA) involves depositing a thin, adherent layer of PGA onto the electrode surface. A step-by-step procedure was followed for PGA modification, along with key the optimization of glassy carbon surface.

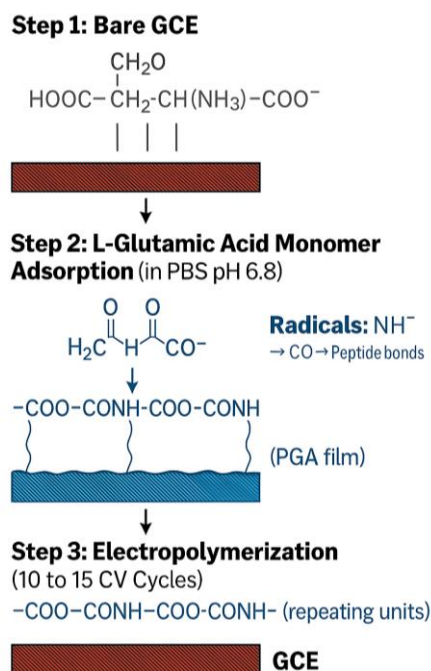
To clean the surface of glassy carbon electrode, the surface of the electrode polished with 0.3 μ alumina and rinsed it with deionized water. Then the electrode was immersed in deionized water and sonicated for 30 s. The surface was wiped with lint free tissue and checked the surface visually to make sure that it was cleaned properly. 1 mg/mL of PGA solution in phosphate buffer with pH 6.8 was prepared and 10 mL of the solution was taken in the electrochemical cell. N₂ Purging was done to remove dissolved oxygen and ensure inert atmosphere. Then the cleaned glassy carbon electrode in PGA solution was immersed for electrodeposition.

Electrochemical pretreatment of GCE was executed by applying potential +1.8 V for about 300 seconds. After that, CV was performed at potential window -1.5 V to $+2.0$ V with scan rate 100 mV/s for 10 to 15 cycles. Finally, activated modified electrode was checked by scanning a single CV within potential window -1.0 V to $+2.0$ V and with a scan rate 50 mV/s. The modified electrode was always rinsed prior to each electrochemical run.

After modification, a thick layer of glossy mirror like film was observed at the glassy carbon electrode surface. In order to confirm that the glassy carbon electrode (GCE) has been modified properly with polyglutamic acid (PGA), we used a combination of electrochemical, spectroscopic, and microscopic techniques.

The material on the electrode was characterized by infra-red (IR) spectroscopy and scanning electron microscopic (SEM) data. A comparative study was also conducted between bare and modified electrode response using CV and electrochemical impedance (EIS) spectra. A schematic representation of the whole process can be shown by scheme 3.1.

3.1.1 Schematic representation of electrode modification



Scheme 3.1 Schematic illustration of electro-polymerization of *L*-glutamic acid onto GCE

Comparative study using CV is shown in Figure 3.1. EIS study details are shown in Figure 3.2. Characterization by IR of the material on the electrode surface is shown in Figure 3.3 and Figure 3.4. The SEM image of the bare and modified electrode surface was given in Figure 3.5.

3.1.2 Characterization of modified electrode using CV

To see the impact of modification of the working electrode, voltammograms were overlaid obtained from 1 mM potassium ferrocyanide in 0.1 M KCl solution using bare glassy carbon electrode and PGA modified glassy carbon electrode. The CV responses are shown in Figure 3. 1.

From the voltammograms (Figure 3. 1) it has been observed that, the current responses obtained using PGA modified electrode is higher than the bare electrode and also the potential was shifted towards more anodic position indicating catalytic activity of the PGA modified electrode surface.

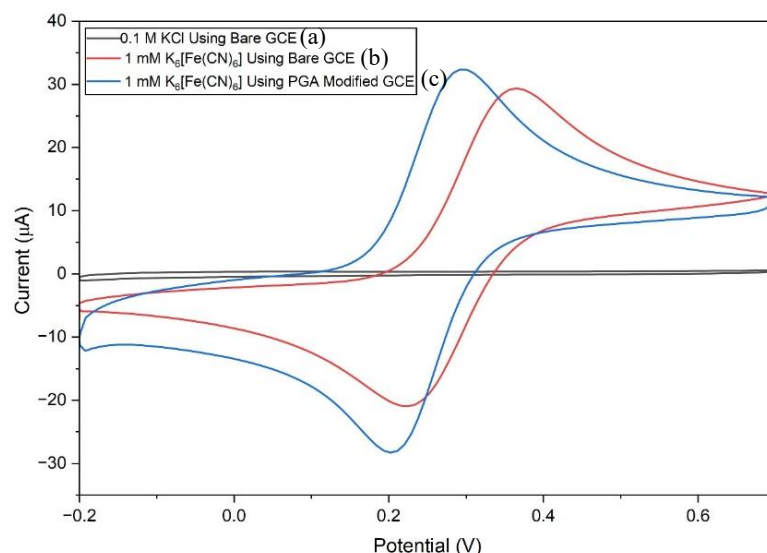


Figure 3. 1: Electrochemical response of potassium ferrocyanide in aqueous 0.1 M KCl solution using bare and PGA modified glassy carbon electrode.

a) CV obtained from 0.1 M KCl solution as blank at 50 mV/s scan rate. b) CV obtained from 1 mM potassium ferrocyanide in aqueous 0.1M KCl solution using bare glassy carbon electrode at 50 mV/s scan rate c) CV obtained from 1 mM potassium ferrocyanide in aqueous 0.1M KCl solution using PGA modified glassy carbon electrode at 50 mV/s scan rate.

3.1.3 Characterization of modified electrode using EIS

To see the impact of modification of the working electrode, electrochemical impedance spectroscopy measurement was taken from 1 mM potassium ferrocyanide in aqueous 0.1M KCl solution using both bare and modified electrode. The Nyquist plots are shown below-

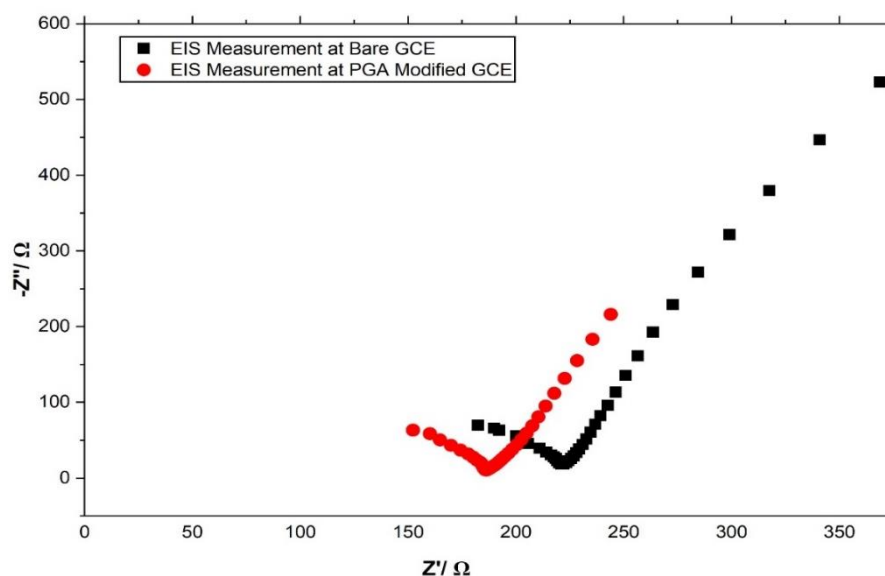


Figure 3. 2: Nyquist plots obtained with bare GCE and PGA modified GCE

From both the Nyquist plots it has been observed that, half semi-circle formed for both bare and modified electrode after applying 1 MHz frequency which would become full semi-circle if extrapolated or increased the frequency. Besides, it is evident the Nyquist plot obtained from modified electrode has been shifted to left which indicates enhance conductivity as well as sensitivity after surface modification.

3.1.4 Characterization of modified electrode using FT-IR

As a part of spectroscopic evaluation of electrode modification, we used FT-IR technique to see the wavenumber of the functional groups in glutamic acid and the materials collected from the glassy carbon electrode surface, where assumed electro-polymerization happened between PGA and GCE. IR spectra of glutamic acid and the materials collected from the glassy carbon electrode surface are shown in Figure 3.3 and 3.4.

From IR spectrum, characteristic peaks are detected, which confirms the adherence of PGA on to the surface of glassy carbon electrode. The key differences in FTIR spectra of *L*-glutamic acid and the PGA modified glassy carbon electrode layer collected from the surface are represented in Table 3.1 and 3.2 and a key difference was presented in Table 3.3.

Table 3.1: Characteristic peaks from the IR spectrum of *L*-glutamic acid (monomer)

Peak (cm ⁻¹)	Identification
~3080–3150	N–H stretch (amine/zwitterion)
~1720 (sharp)	C=O stretch of protonated –COOH (carboxylic acid dimer)
~1600 and 1400	Zwitterion peaks: asymmetric/symmetric COO ⁻ stretches
~1500–1580	N–H band (NH ₃ ⁺ deformation)
~1200–1300	C–O stretch of –COOH

IR Spectra of *L*-glutamic acid (monomer)

The chemical structure of glutamic acid monomer is H₂N–CH(CH₂CH₂COOH)–COOH (zwitterionic form in solid state). The characteristic peaks from the IR spectrum are captured described in Table 3.2.

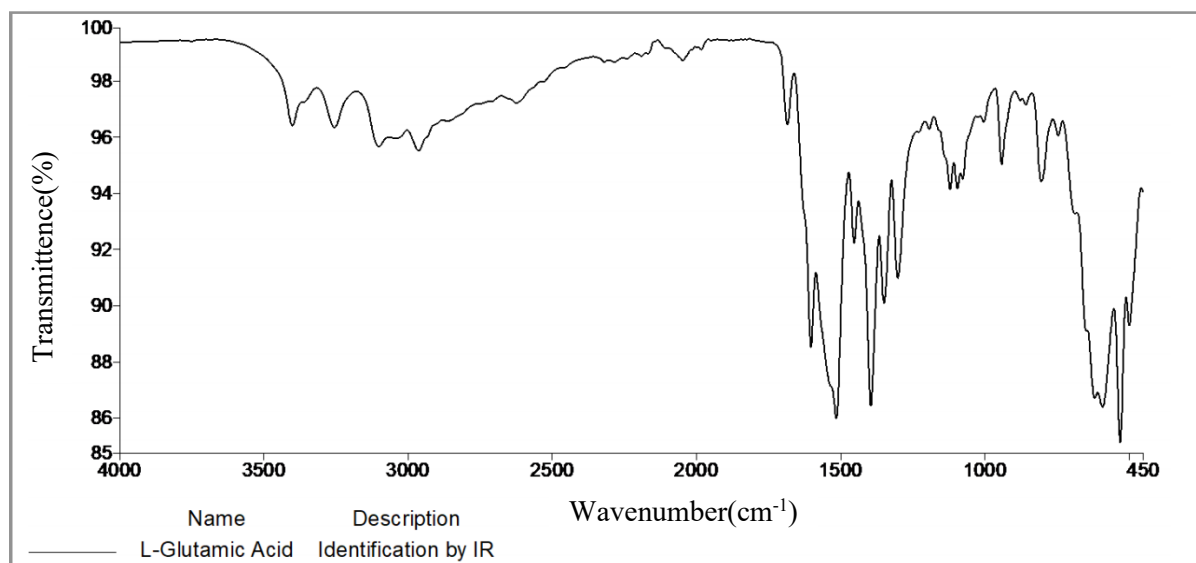


Figure 3. 3: IR spectrum of *L*-glutamic acid

IR spectra of polyglutamic acid (PGA)-polymer

The chemical structure consists of repeating units with amide bonds ($-\text{NH}-\text{CH}(\text{CH}_2\text{CH}_2\text{COOH})-\text{CO}-$)_n. The characteristic peaks are listed in Table 3.2.

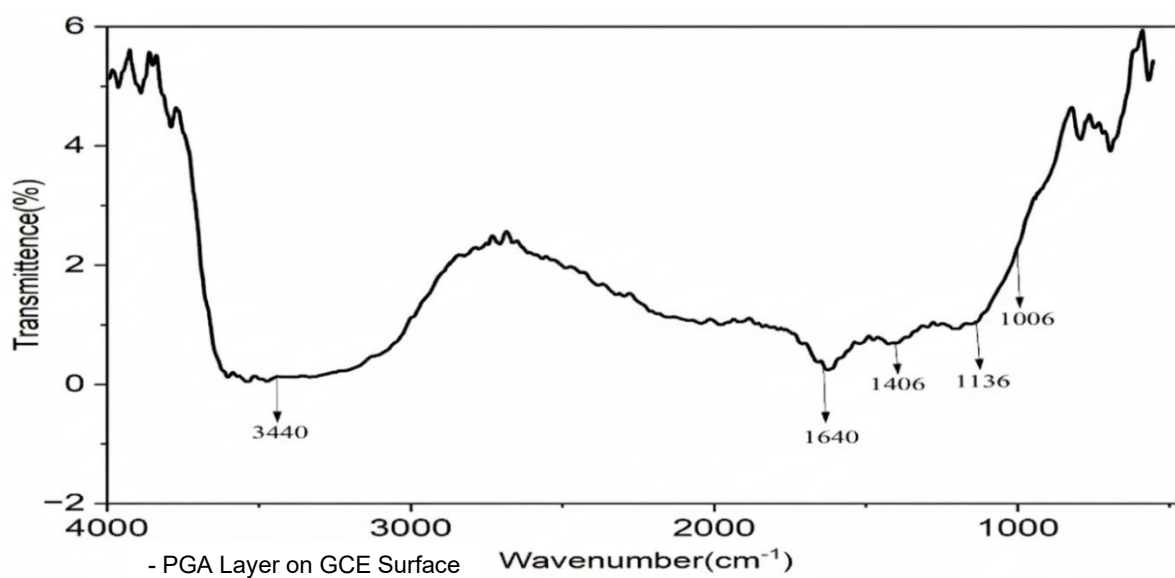


Figure 3. 4: IR spectrum of poly-glutamic acid modified glassy carbon electrode layer collected from the surface

Table 3.2: Characteristic peaks from the IR spectrum polyglutamic acid (PGA)-polymer

Peak (cm ⁻¹)	Identification	vs. Monomer
~3400-3500 (broad)	N–H stretch (amide A)	Broader than monomer
~1640–1660	Amide I: C=O stretch (peptide bond)	Replaces monomer's 1720 cm ⁻¹
~1400–1500	Amide II: N–H bend + C–N stretch	Absent
~1550–1600	Carboxylate (–COO ⁻) asymmetric stretch	Weaker than monomer (side chains)
~1400–1450	Carboxylate symmetric stretch	Weaker than monomer

Key data obtained from both the spectra are tabulated in Table 3.3 for direct comparison between *L*-glutamic acid (monomer) and polyglutamic acid (PGA)-polymer.

Table 3.3: Direct comparison table between *L*-glutamic acid (monomer) and polyglutamic acid (PGA)-polymer

Peak Type	Monomer (<i>L</i> -Glutamic Acid)	PGA Polymer
N–H Stretch	~3080–3150 (sharp)	~3440 (broad, amide A)
C=O Stretch	1720 (carboxylic acid)	1640 (amide I)
Carboxylate	1600/1400 (zwitterion)	Weaker (side chains)
Amide II Band	Absent	1400–1500 (key identifier)
C–O Stretch	~1200–1300 (strong)	Weak/merged with amide II

Based on the above data comparison it can be concluded that, the peak observed in monomer 1720 cm⁻¹ disappears in PGA which indicates carboxyl groups converted to amide bonds. Amide I (1640 cm⁻¹) and Amide II (1400-1500 cm⁻¹) appeared which indicates peptide backbone formed. Besides, N–H stretch broadens (3440 cm⁻¹) due to H–bonding in polymer matrix. So, it is confirmed the polymerization happened during electrode medication by applying potential.

3.1.5 Surface characterization of modified electrode using SEM

As a part of surface characterization of PGA modified glassy carbon electrode, used SEM techniques to see the morphology. SEM image of the materials collected from the glassy carbon electrode surface after modification with PGA is shown in Figure 3.5.

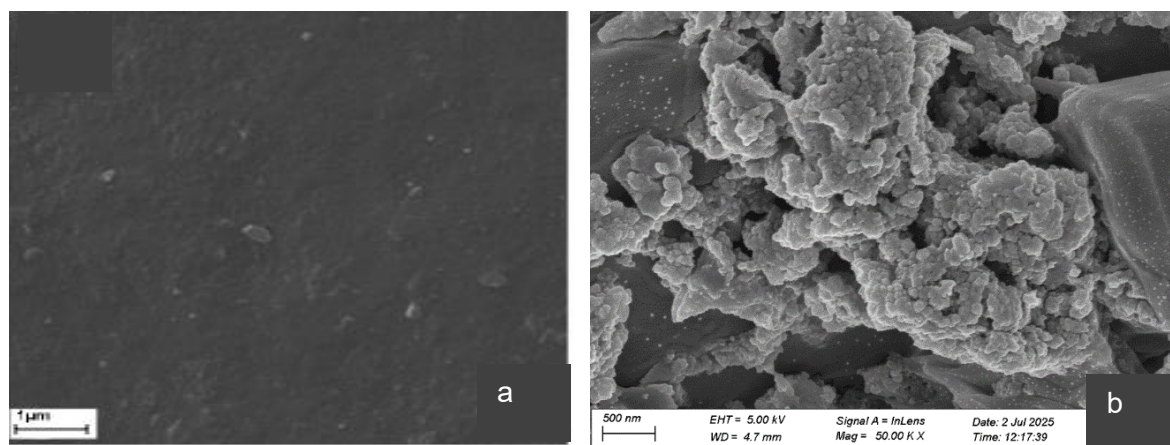


Figure 3. 5: SEM image of a) surface of bare glassy carbon electrode b) surface layer of PGA modified glassy carbon electrode

From the image it has been observed clearly that, there is a morphological change in the surface which conforms surface modification.

3.1.6 Summary of characterization study of modified electrode

Based on the above-mentioned different techniques like CV, EIS, IR and SEM data, it can be summarized that the modification of glassy carbon electrode using PGA was done successfully.

3.2 Study of Cefitibuten

Ceftibuten belongs to the third generation of cephalosporin antibiotics and is clinically employed in the management of various bacterial infections. Its therapeutic applications include treatment of acute bacterial exacerbations of chronic bronchitis (ABECB), acute otitis media, pharyngitis, and tonsillitis. Additionally, it is indicated for respiratory tract infections such as pneumonia, as well as infections affecting the urinary tract, gastrointestinal tract including enteritis and gastroenteritis¹⁵²⁻¹⁵⁴. The molecular structure of ceftibuten is illustrated in Figure 3.6.

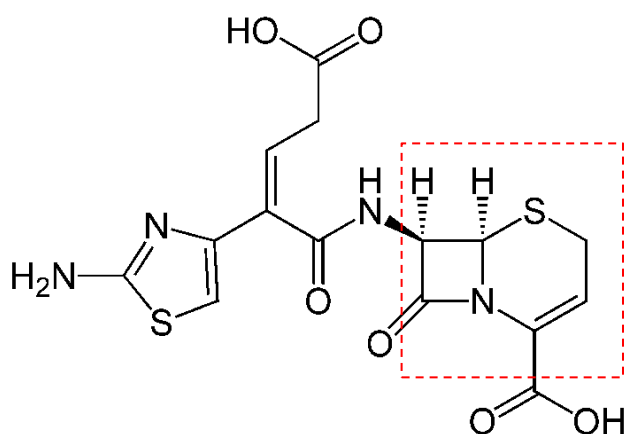


Figure 3. 6: Chemical structure of ceftibuten

As the objective of the study is to determine the antibiotic resistance using electrochemical techniques so, it is needed to develop suitable and easy to handle electrochemical methods which can be applied for ceftibuten assay determination in different conditions. Specifically, the method should be capable to determine the content of ceftibuten in pure state and similarly when spiked with the blood sample. Accordingly, and with the availability among various techniques, we applied CV, LSV and DPV.

In this study from section 3.2.1 to section 3.2.3, described the detailed explanation of the procedure of method development on the assay or content determination of ceftibuten in pure state. In section 3.2.4, mentioned the details about the study of ceftibuten when spiked with blood samples. And, finally in section 3.2.5, explained the study outcome of in vitro study of antibiotic resistance using β -lactamase enzyme to see the degradation pattern after adding it within the solution of ceftibuten having blood serum in phosphate buffer pH 6.8.

3.2.1 Cyclic voltammetric study of ceftibuten

CV study was carried out using PGA modified GCE in phosphate buffer solution at different pH from 4.5 to 7.4 within the potential window from -0.2 V to $+1.5$ V at various scan rates and various concentrations. The solubility of ceftibuten was checked in different pH of phosphate buffer and found, it is soluble in acidic, basic and neutral pH of phosphate buffer solution. Stock solution was prepared in phosphate buffer solution.

As a part of electrochemical set up, PGA modified GCE was used as working electrode, Ag/AgCl was used as reference electrode and platinum electrode was used counter electrode and the potential window maintained from -0.2 V to $+1.5$ V.

3.2.1.1 Study of redox behavior of ceftibuten in phosphate buffer at different pH

To observe the redox behavior of ceftibuten, a series of cyclic voltammograms were taken with 20 ppb ceftibuten at 50 mV/s scan rate within the switching potential -0.2 V to $+1.5$ V vs. Ag/AgCl, which are shown in Figure 3.7. Peak current and pH variation are tabulated in Table 3.4.

Cyclic voltammograms were taken from 20 ppb ceftibuten solution within a potential range of -0.2 V to 1.5 V, a glassy carbon electrode modified with poly glutamic acid was used as working electrode. A platinum (Pt) wire was used as the counter electrode and an Ag/AgCl electrode was used as the reference electrode.

From Figure 3.7, we can see that, there is a single anodic peak found for each of the voltammogram between +0.7 V to +0.9 V which is an oxidation peak could be happened due to the oxidation of the β -lactam ring or sulfur-containing side chains. And, an irreversible reaction happened during this electrochemical process.

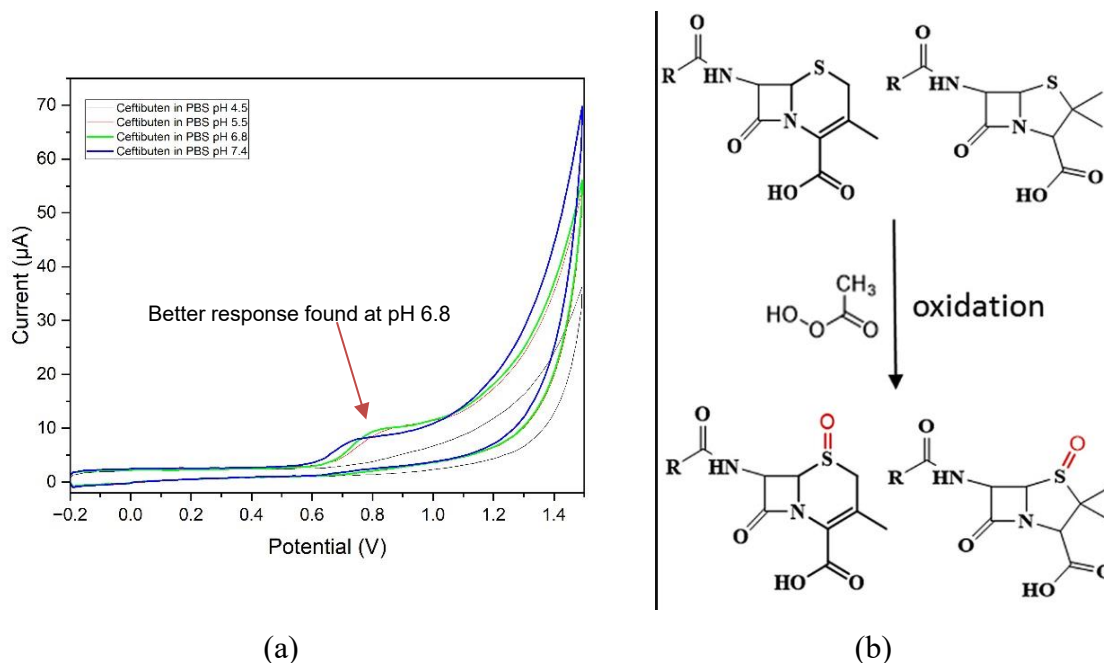


Figure 3. 7: Electrochemical responses and reactions happened during CV run.

a) Cyclic voltammograms of 20 ppb ceftibuten in phosphate buffer at different pH using PGA modified GCE b) Electrochemical reaction happened in β -lactam ring when applied potential.

Analysis of the overlaid plot revealed an enhanced electrochemical response (Table 3.4) in phosphate buffer at pH 6.8. From the voltammograms, better response found at pH 6.8 and the peak current found 9.88 μ A.

Table 3.4: Change of peak current with the variation of pH for the response of 20 ppb ceftibuten in PBS at 50 mV/s scan rate

pH	Peak current (μ A)
4.5	3.62
5.5	8.43
6.8	9.88
7.4	7.96

3.2.1.2 Study of minimum detection level of ceftibuten using PGA modified GCE

Once optimized the suitable pH of phosphate buffer pH 6.8, studied the minimum detection level of ceftibuten using PGA modified GCE. For this purpose, CV were taken for 1 ppb, 5 ppb, 10 ppb and 20 ppb of ceftibuten solutions in PBS at pH 6.8. The voltammograms are overlaid and shown in Figure 3.8.

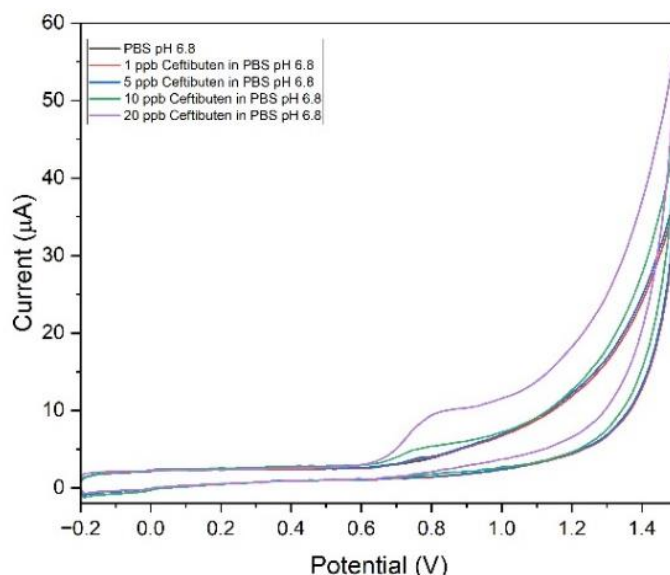


Figure 3. 8: Cyclic voltammograms of different concentrations of ceftibuten in PBS with pH 6.8 using PGA modified GCE at 50 mV/s scan rate

The CV response shows a single irreversible peak at around 0.75 V. Based on the data obtained from voltammograms, response (peak current) is observed even from 1 ppb of ceftibuten solution. So, it is assumed that it would be capable to detect a trace level of ceftibuten if present in chemical or biological sample, like blood.

3.2.1.3 Accuracy check of CV method for ceftibuten determination

Cyclic voltammograms (Figure 3.9) were taken for 10 ppb, 15 ppb, 20 ppb, 25 ppb and 30 ppb solutions of ceftibuten in PBS with pH 6.8 using PGA modified glassy carbon electrode.

Data obtained from the voltammograms are captured in Table 3.5 and plotted graph in Figure 3.10. From linearity curve, correlation coefficient found above 0.995 which shows a satisfactory accuracy and capability of detection. The peak potential is slightly shifted toward the anodic position with increasing the concentration of ceftibuten indicating irreversible charge transfer of ceftibuten.

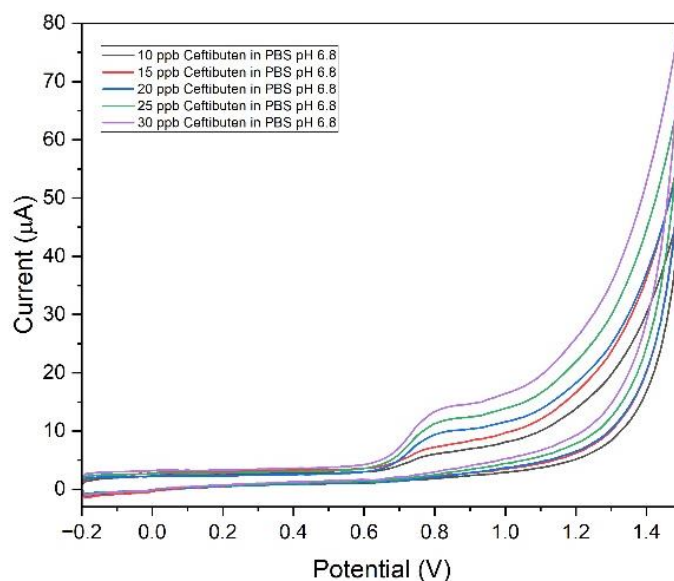


Figure 3. 9: Electrochemical responses (CV) of 10 ppb, 15 ppb, 20 ppb, 25 ppb and 30 ppb solutions of ceftibuten in PBS with pH 6.8 at 50 mV/s scan rate

Table 3.5: Electrochemical responses (peak current) of different concentration of ceftibuten at 50 mV/s scan rate

Concentration of Ceftibuten (ppb)	Peak Current (μA)
10	5.69
15	7.18
20	9.63
25	11.56
30	13.68

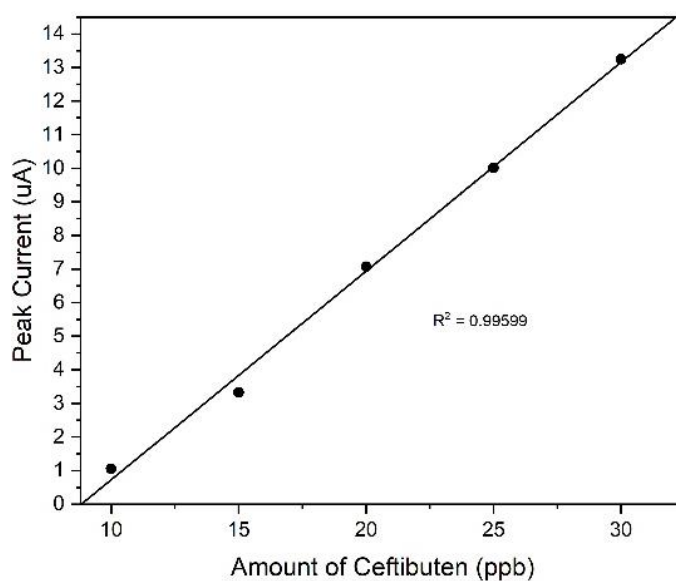


Figure 3. 10: Variation showing linearity of CV method based on different concentration of ceftibuten

3.2.1.4 Effect of scan rate variation on ceftibuten

Cyclic voltammograms were taken from 20 ppb ceftibuten in phosphate buffer solution pH 6.8 using PGA modified glassy carbon electrode by different scan rate 25, 50, 75 and 100 mV/s within the potential window -0.2 V to $+1.5$ V. Cyclic voltammograms are shown in Figure 3.11 and peak current with scan rate variation are tabulated in Table 3.6. The peak current was increased with increasing scan rate as it is expected.

The peak current data obtained from the scan rate variation are tabulated in Table-3.6. The calibration plot (Figure-3.12) shows a strong correlation coefficient value which indicates the robustness of the CV test method for ceftibuten determination

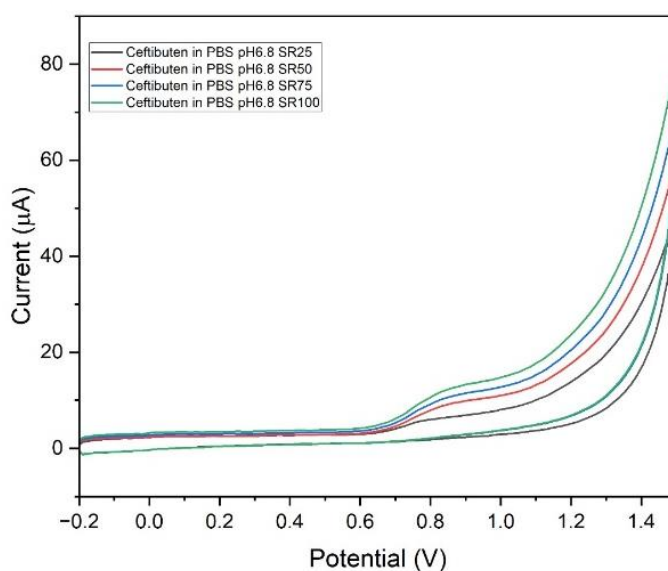


Figure 3. 11: Cyclic voltammograms of 20 ppb ceftibuten in PBS with pH 6.8 at 25, 50, 75 and 100 mV/s scan rate

Table 3.6: Electrochemical responses with scan rate variation from 20 ppb ceftibuten in PBS with pH 6.8

Scan Rate (ν)/mV/s	$\sqrt{\nu}$	Peak Current (μ A)
25	5.0	6.43
50	7.07107	8.78
75	8.66025	10.56
100	10	12.63

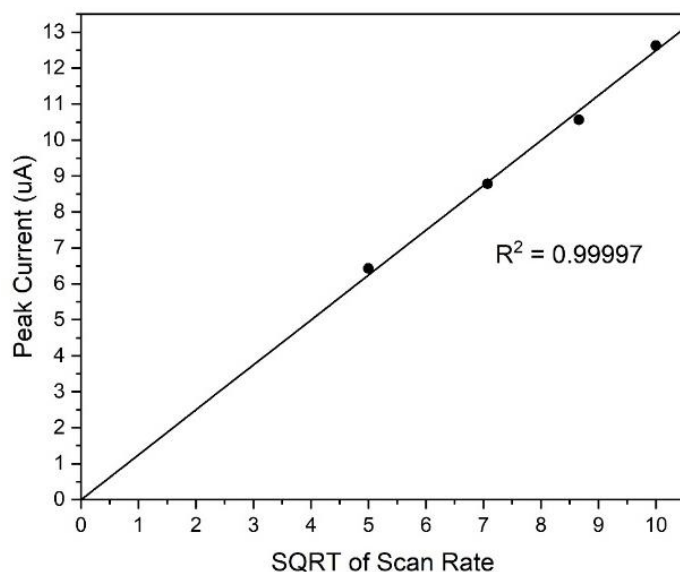


Figure 3. 12: Variation of peak current with SQRT of scan rate for 20 ppb ceftibuten in PBS

So, based on the above all studies data it can be inferred, PGA modified glassy carbon electrode can determine the ceftibuten reproducibly using CV in phosphate buffer solution pH 6.8.

3.2.2 Study of ceftibuten with linear sweep voltammetry (LSV) technique

Linear sweep voltammetric study of ceftibuten was carried out using PGA modified GCE in phosphate buffer solution pH 6.8 within the potential window from +0.3 V to +1.3 V at various scan rates and concentrations.

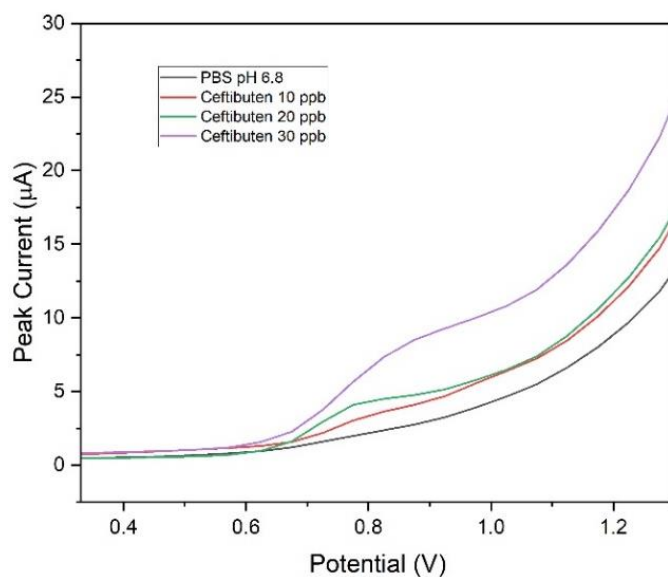


Figure 3. 13: Electrochemical responses (LSV) of 10 ppb, 20 ppb and 30 ppb solutions of ceftibuten in PBS with pH 6.8 at 50 mV/s scan rate

From the voltammograms (Figure 3.13) it has been observed that, PGA modified GCE is showing response in LSV technique also. However, the accuracy of LSV technique is not prominent as like cyclic voltammetry.

3.2.3 Study of ceftibuten using differential pulse voltammetry

Differential pulse voltammetric study of ceftibuten was conducted using a PGA-modified glassy carbon electrode in phosphate buffer solution pH 6.8. The electrochemical analysis was performed within a potential window of +0.1 V to +1.3 V, varying both the scan rates and analyte concentrations. The stock solution of ceftibuten was prepared in PBS with pH 6.8, and all experiments were carried out under identical pH conditions of pH 6.8.

From the voltammograms represented in Figure 3.14, it was observed that with different concentrations of ceftibuten solutions a prominent response with PGA modified GCE at 50 mV/s scan rate was found. This indicates a possibility to detect a trace level of ceftibuten in real sample (blood sample), which will help later to determine antibiotic resistance as well.

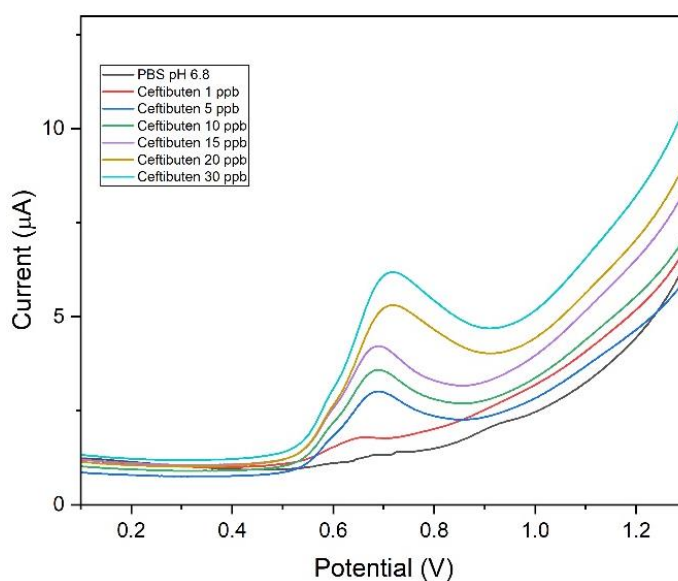


Figure 3. 14: Differential pulse voltammograms of 1 ppb, 5 ppb, 10 ppb, 15 ppb, 20 ppb and 30 ppb solutions of ceftibuten in PBS with pH 6.8 at 50 mV/s scan rate

3.2.4 Recovery study of ceftibuten in blood samples

To conduct study using human blood sample, ethical clearance was taken from the Faculty of Biological Science, University of Dhaka as approved by ethical committee. Afterward, blood samples were collected from volunteers, along with relevant medical history and physical conditions data essential to the study.

The study was designed in a sequential step like, 10 mL phosphate buffer pH 6.8 was taken in a cell. Blank run has been captured with CV and DPV techniques using PGA modified GCE at 50 mV/s scan rate. After that, 100 μ L blood serum was added in phosphate buffer solution and CV and DPV were taken using PGA modified GCE. Then, different concentrations of ceftibuten solutions were added in the phosphate buffer containing blood serum and again CV and DPV experiments were performed using PGA modified GCE. Finally, a solution having 20 ppb ceftibuten kept for more than 24 hours in room temperature at around 35°C to 37°C and CV and DPV experiments were performed using PGA modified GCE at a regular interval up to 24 hours.

3.2.4.1 Study of ceftibuten and blood sample collected from human volunteer-1 by CV using PGA modified GCE

As a part of study, collected blood samples from human volunteers. Then, centrifuged it to separate blood serum and used blood serum in study. In this study, the volunteers blood group is A (+)-ve with body weight 78 kg and age 26 years. The volunteer was the resident of Mirpur, Dhaka.

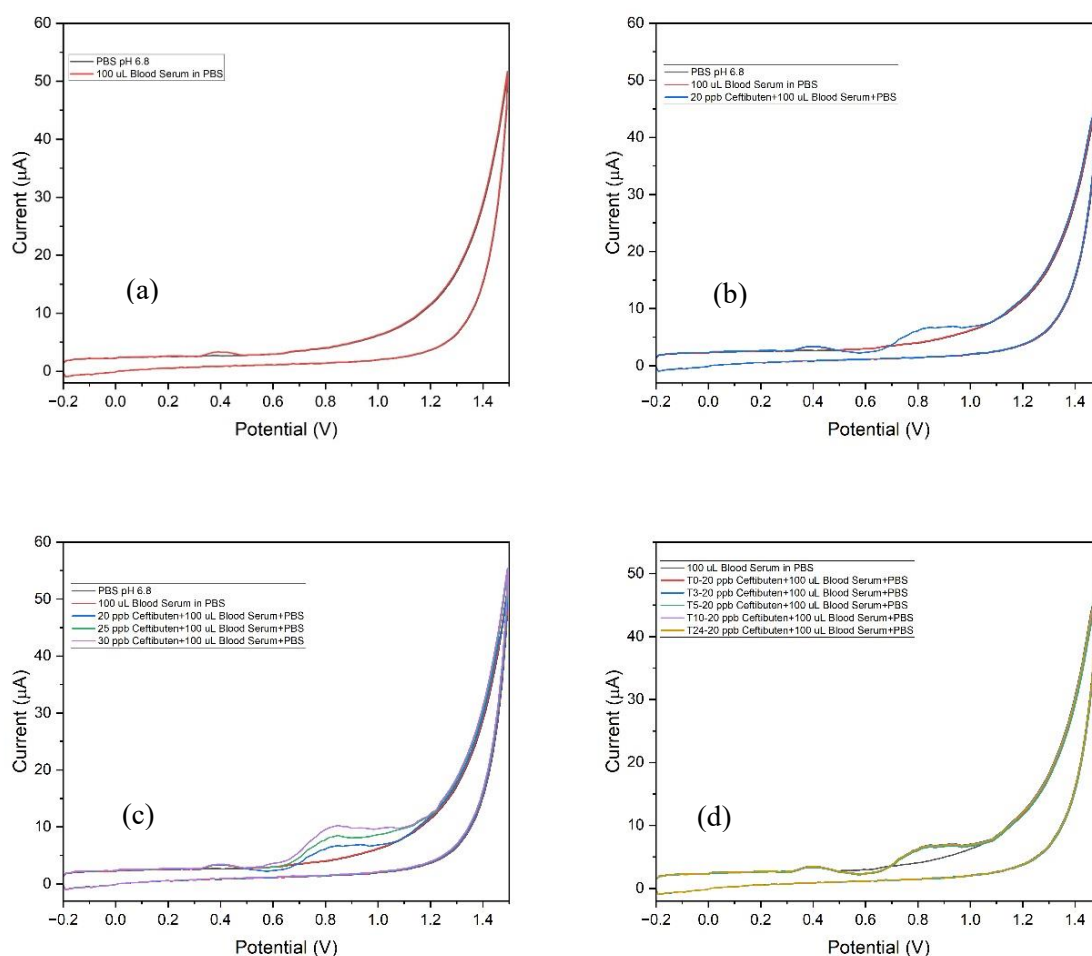
Cyclic voltammetric study of ceftibuten and blood serum was conducted using PGA-modified glassy carbon electrode in phosphate buffer solution pH 6.8. The electrochemical analysis was performed within a potential window of -0.2 V to $+1.5$ V at 50 mV/s scan rate. Blank CV run was recorded from 10 mL of phosphate buffer pH 6.8 solution. Then, ran CV scan by adding 100 μ L blood serum. Afterwards, added different concentrations of ceftibuten within it and acquired CV. Also, acquired CV measurements up to 24 hours at a regular interval.

The voltammograms reveal distinct current signal peaks in blood serum at various potentials, yet no interference is observed at the potential corresponding to ceftibuten $+ 0.830$ V. The current responses obtained from different concentrations of ceftibuten shows similar accuracy of method observed during the study in phosphate buffer pH 6.8 in absence of blood serum which confirms there is no interference from blood serum. From Figure 3.15 (d) it has been observed that, at different time intervals, the peak current responses remain nearly constant.

The data (Table 3. 7) shows the stability of both solution and modified electrode over 24 hours. It also indicates, subject volunteer blood doesn't have any bacteria as well as resistant bacteria which can generates β -Lactamase. So, current response remains almost consistent of ceftibuten over a period of time.

Table 3. 7: Peak current response from cyclic voltammograms with time

Volunteer	Potential (V)	Current (μA)					Difference in Current from T_0 to T_{24} (μA)	Remarks
		T_0	T_3	T_5	T_{10}	T_{24}		
Volunteer-1	+0.8307	6.71	6.72	6.71	6.70	6.68	-0.03	Current response consistent

**Figure 3. 15:** Electrochemical responses recorded from blank, blood serum and different concentration of ceftibuten.

a) Cyclic voltammograms of PBS with pH 6.8 as blank and 100 μL blood serum in PBS with pH 6.8 b) Cyclic voltammograms of 20 ppb ceftibuten in PBS with pH 6.8 with 100 μL blood serum c) Cyclic voltammograms of 20, 25 and 30 ppb ceftibuten in PBS with pH 6.8 with 100 μL blood serum d) Cyclic voltammograms of 20 ppb ceftibuten in PBS with pH 6.8 with 100 μL blood serum at different time interval i.e. initial, after 3, 5, 10 and 24 hours.

3.2.4.2 Study of ceftibuten and blood sample collected from human volunteer-1 by DPV using PGA modified GCE

Differential pulse voltammetric study of ceftibuten and blood serum was conducted using PGA-modified glassy carbon electrode in phosphate buffer solution pH 6.8 within the potential window of +0.1 V to +1.3 V at 50 mV/s scan rate.

Measurement of blank was acquired from 10 mL of phosphate buffer solution pH 6.8. Then, ran DPV scan by adding 100 μ L blood serum. After that, added different concentrations of ceftibuten within it and acquired DPV. Also, acquired DPV measurements up to 24 hours at a regular interval.

The voltammograms (Figure 3. 16) reveal distinct current signal from blood serum at various potentials and no interference is observed at the same potential corresponding to ceftibuten + 0.888 V. The current responses obtained from different concentrations of ceftibuten shows similar accuracy of method observed during the study in phosphate buffer pH 6.8 in absence of blood serum which confirms there is no interference from blood serum.

Here also it can be noted that at different time intervals, the peak current response remains nearly unchanged (Table 3.8).

Table 3.8: Peak current response from differential pulse voltammograms with time

Volunteer	Potential (V)	Current (μ A)					Difference in Current from T ₀ to T ₂₄ (μ A)	Remarks
		T ₀	T ₃	T ₅	T ₁₀	T ₂₄		
Volunteer-1	+0.888	3.952	3.952	3.951	3.952	3.951	−0.001	Response remains almost consistent

DPV data also shows the stability of both solution and modified electrode over 24 hours.

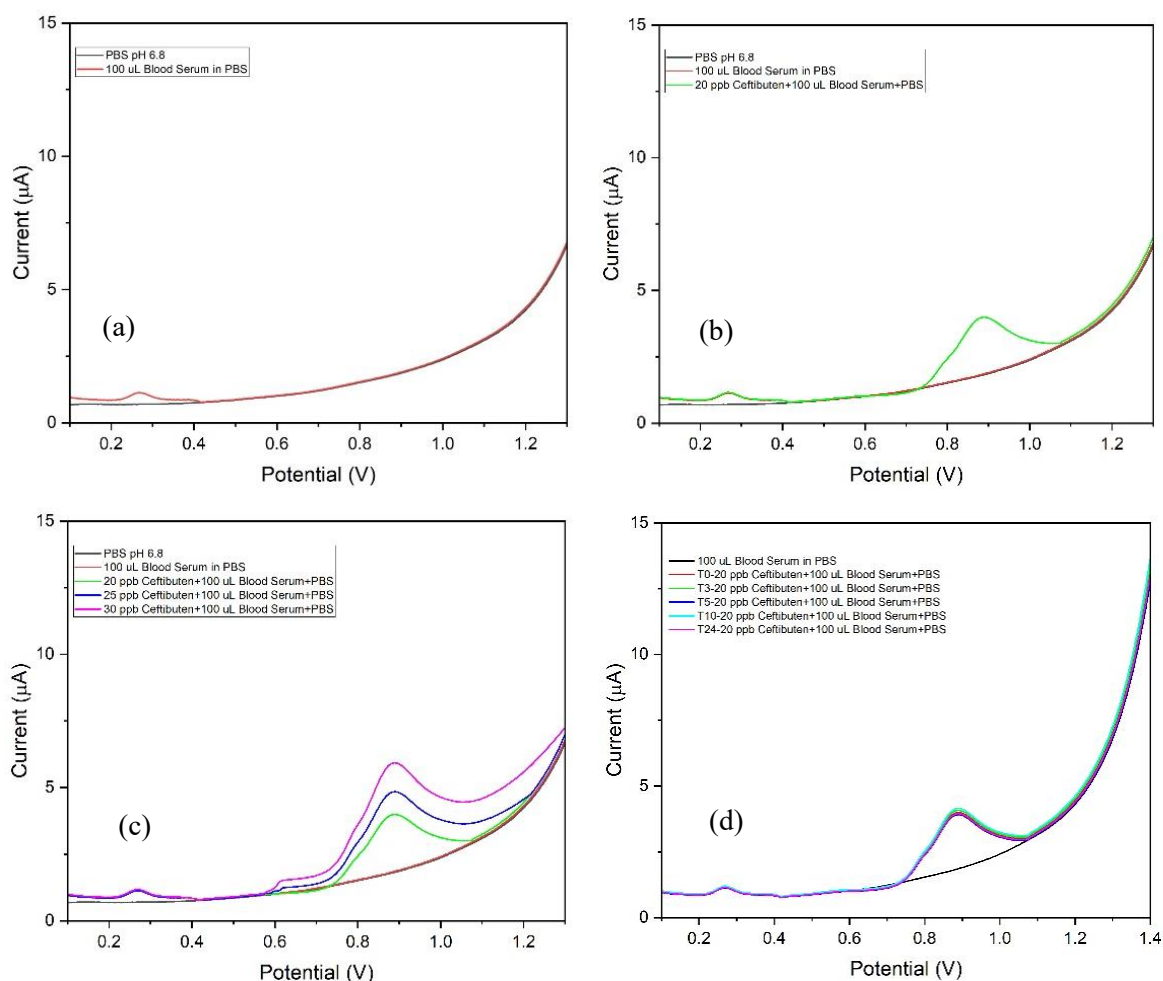


Figure 3. 16: Electrochemical responses recorded from blank, blood serum and different concentration of cefibuten.

a) Voltammograms of PBS with pH 6.8 as blank and 100 μL blood serum in PBS with pH 6.8 b) Voltammograms of 20 ppb cefibuten in PBS with pH 6.8 with 100 μL blood serum c) Voltammograms of 20, 25 and 30 ppb cefibuten in PBS with pH 6.8 with 100 μL blood serum d) Voltammograms of 20 ppb cefibuten in PBS with pH 6.8 with 100 μL blood serum at different time interval (initial, 3, 5, 10 & 24 hours) up to 24 hours.

3.2.4.3 Study of cefibuten and blood sample collected from human volunteer-2 by CV using PGA modified GCE

Blood sample was collected from the volunteer who is a male of 70 years old resident of Hajaribag, Dhaka. His blood group is A (+)-ve and body weight was 70 kg.

The study of cefibuten and blood serum was conducted using PGA-modified glassy carbon electrode in phosphate buffer solution pH 6.8. The analysis was conducted within a potential window of -0.2 V to $+1.5\text{ V}$ at 50 mV/s scan rate. Blank CV run was recorded from 10 mL

of phosphate buffer pH 6.8 solution. Then, ran CV scan by adding 100 μ L blood serum. Afterwards, added different concentrations of ceftibuten within it and acquired CV. Also, acquired CV measurements up to 24 hours at a regular interval.

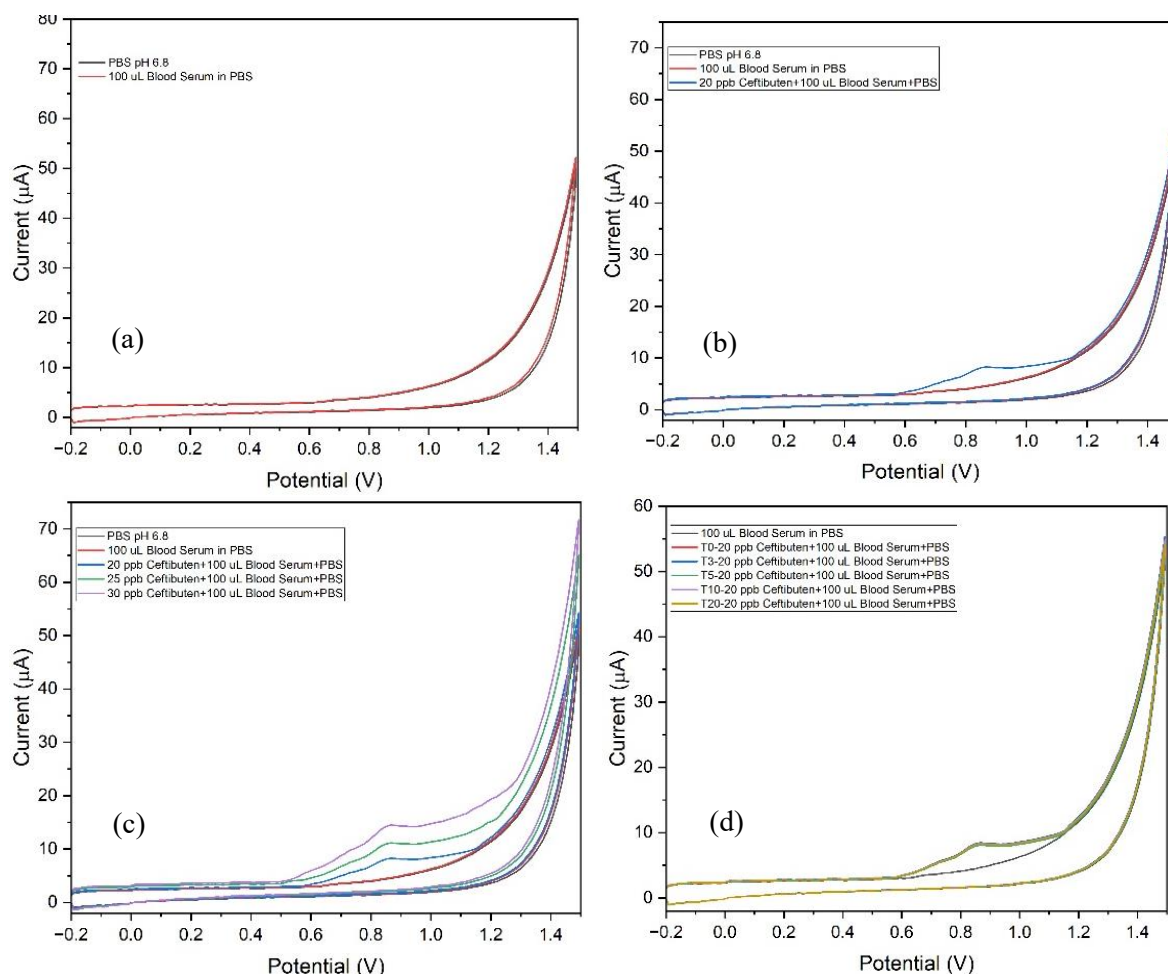


Figure 3. 17: Electrochemical responses recorded from blank, blood serum and different concentration of ceftibuten.

a) Cyclic voltammograms of PBS with pH 6.8 as blank and 100 μ L blood serum in PBS with pH 6.8 b) Cyclic voltammograms of 20 ppb ceftibuten in PBS with pH 6.8 with 100 μ L blood serum c) Cyclic voltammograms of 20, 25 and 30 ppb ceftibuten in PBS with pH 6.8 with 100 μ L blood serum d) Cyclic voltammograms of 20 ppb ceftibuten in PBS with pH 6.8 with 100 μ L blood serum at different time interval i.e. initial, after 3, 5, 10 and 24 hours.

From the voltammograms (Figure 3. 17) observed that, there are some very low signals in blood serum at various potentials, but no interference is observed at the potential corresponding to ceftibuten + 0.840 V. Current responses found from different concentrations of ceftibuten shows similar accuracy of method observed during the study in phosphate buffer pH 6.8 in absence of blood serum which confirms there is no interference from blood serum.

Table 3.9: Peak current response from cyclic voltammograms with time

Volunteer	Potential (V)	Current (μA)					Difference in Current from T_0 to T_{24} (μA)	Remarks
		T_0	T_3	T_5	T_{10}	T_{24}		
Volunteer-2	+0.8401	8.09	8.08	8.09	8.07	8.06	-0.03	Current response remains almost consistent

The data (Table 3.9) shows the stability of both solution and modified electrode over 24 hours. It should be noted that the Figure 3. 17 (d) illustrates the peak current responses at different time intervals remain nearly unchanged.

3.2.4.4 Study of ceftibuten and blood sample collected from human volunteer-2 by DPV using PGA modified GCE

This electrochemical study of ceftibuten and blood serum was conducted using PGA-modified glassy carbon electrode in phosphate buffer solution pH 6.8 within the potential window of +0.1 V to +1.3 V at 50 mV/s scan rate. Measurement of blank was acquired from 10 mL of phosphate buffer solution pH 6.8. Then, ran DPV scan by adding 100 μL blood serum. After that, added different concentrations of ceftibuten within it and acquired DPV. Also, acquired DPV measurements up to 24 hours at a regular interval.

The voltammograms (Figure 3. 18) reveal no distinct current signal from blood serum at various potentials and no interference is observed at the same potential corresponding to ceftibuten + 0.888 V. The current responses obtained from different concentrations of ceftibuten shows similar accuracy of method observed during the study in phosphate buffer pH 6.8 in absence of blood serum which confirms there is no interference from blood serum. It seems across different time intervals, the peak current response remains almost consistent (Table 3.10) shown in Figure 3. 18 (d).

Table 3.10: Peak current response from differential pulse voltammograms with time

Volunteer	Potential (V)	Current (μA)					Difference in Current from T_0 to T_{24} (μA)	Remarks
		T_0	T_3	T_5	T_{10}	T_{24}		
Volunteer-2	0.888	3.914	3.915	3.914	3.915	3.915	0.001	Current remains almost consistent

Here also, the data shows the stability of both solution and modified electrode over 24 hours.

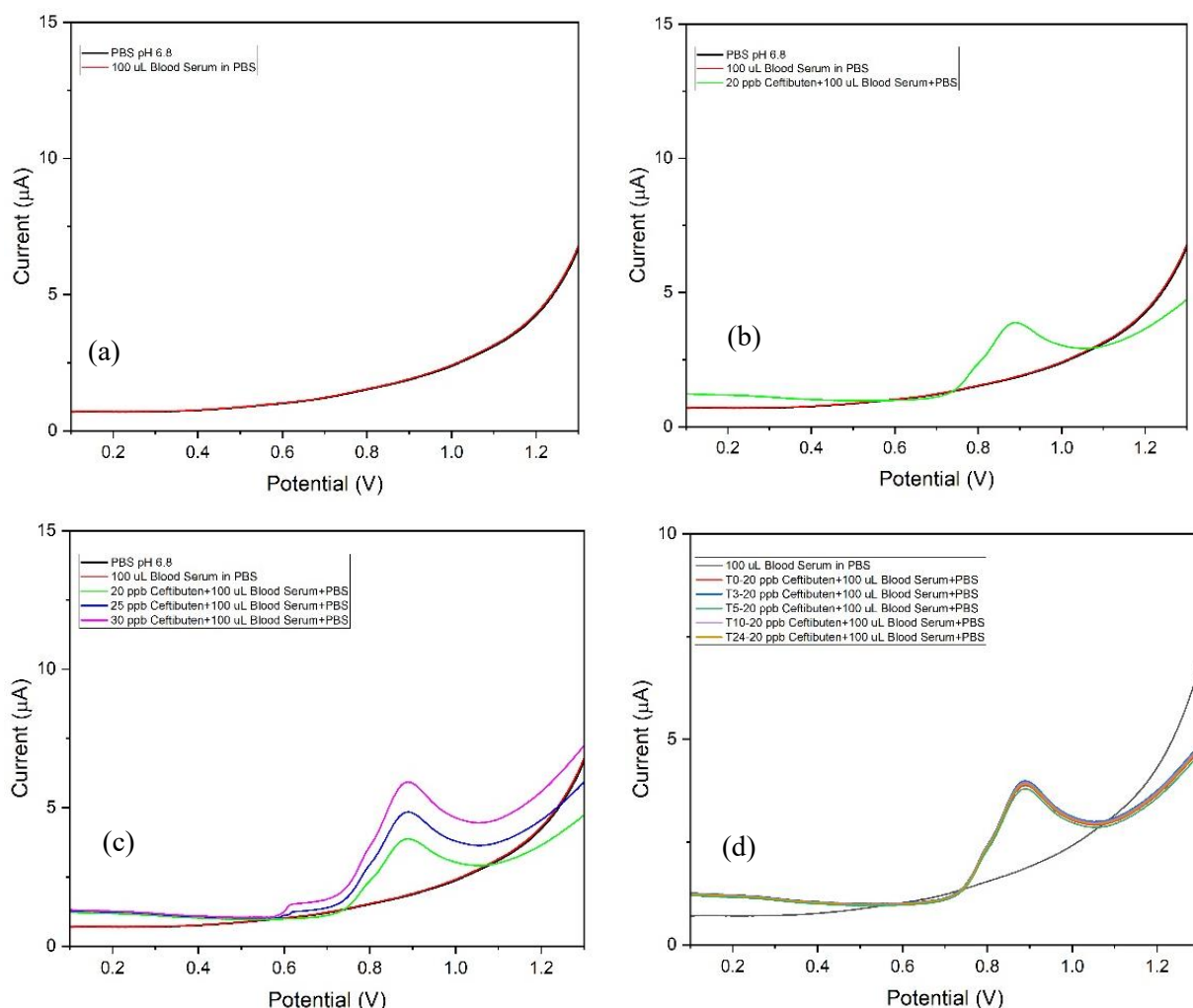


Figure 3. 18: Electrochemical responses recorded from blank, blood serum and different concentration of ceftibuten.

a) Voltammograms of PBS with pH 6.8 as blank and 100 μL blood serum in PBS with pH 6.8 b) Voltammograms of 20 ppb ceftibuten in PBS with pH 6.8 with 100 μL blood serum c) Voltammograms of 20, 25 and 30 ppb ceftibuten in PBS with pH 6.8 with 100 μL blood serum d) Voltammograms of 20 ppb ceftibuten in PBS with pH 6.8 with 100 μL blood serum at different time interval i.e. initial, after 3, 5, 10 hours and up to 24 hours.

3.2.4.5 Study of ceftibuten and blood sample collected from human volunteer-3 by CV using PGA modified GCE

As a part of study, collected blood samples from human volunteers. Then, centrifuged it to separate blood serum and used blood serum in study. In this study, the volunteers blood group is A (+)-ve. His body weight was 64 kg and age was 22 years. The volunteer was the resident of Shahidullah Hall, Dhaka University.

Cyclic voltammetric study of ceftibuten and blood serum was conducted using PGA-modified glassy carbon electrode in phosphate buffer solution pH 6.8. The electrochemical analysis was performed within a potential window of -0.2 V to $+1.5$ V at 50 mV/s scan rate. Blank CV run was recorded from 10 mL of phosphate buffer pH 6.8 solution. Then, ran CV scan by adding 100 μ L blood serum. Afterwards, added different concentrations of ceftibuten within it and acquired CV. Also, acquired CV measurements up to 24 hours at a regular interval.

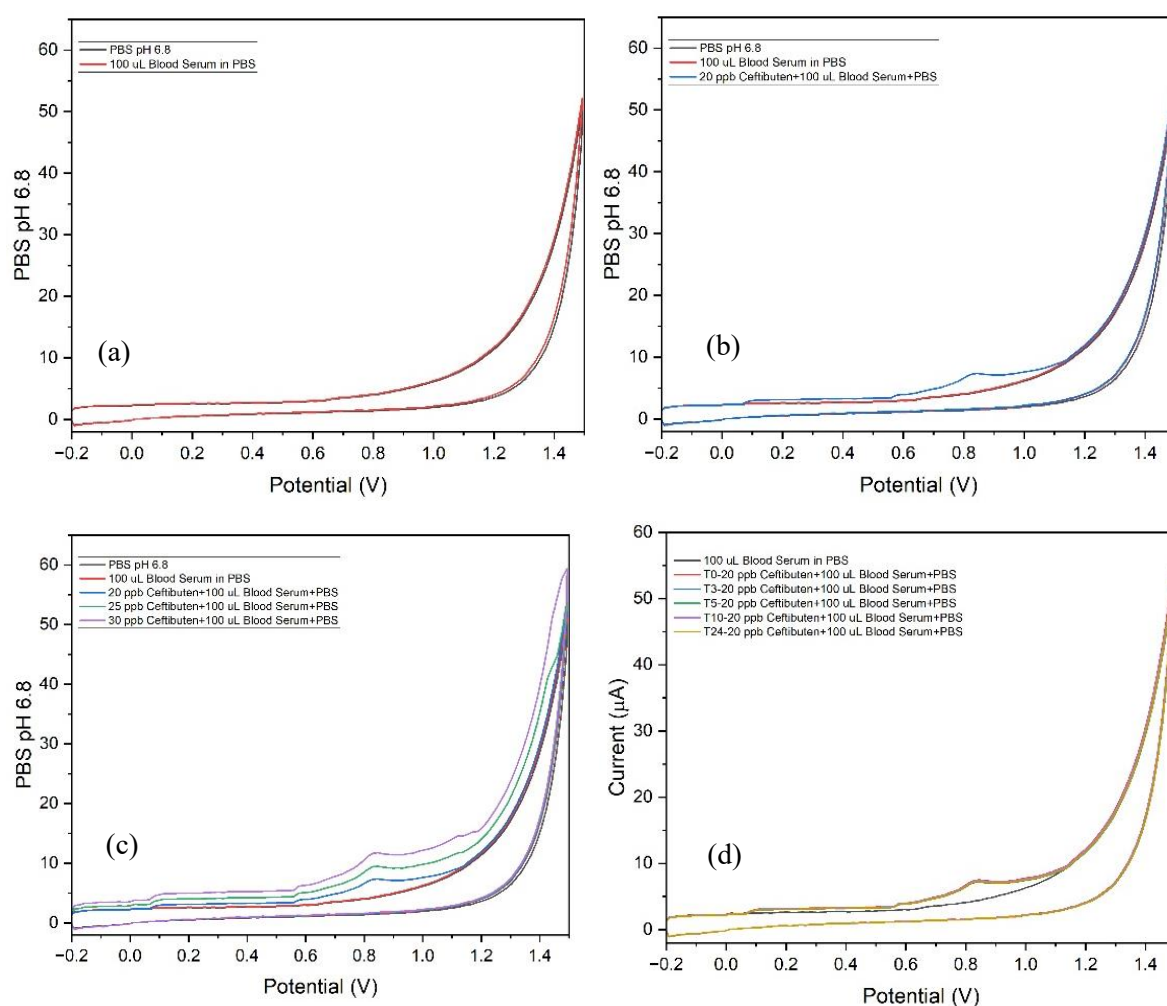


Figure 3. 19: Electrochemical responses recorded from blank, blood serum and different concentration of ceftibuten.

a) Cyclic voltammograms of PBS with pH 6.8 as blank and 100 μ L blood serum in PBS with pH 6.8 b) Cyclic voltammograms of 20 ppb ceftibuten in PBS with pH 6.8 with 100 μ L blood serum c) Cyclic voltammograms of 20 , 25 and 30 ppb ceftibuten in PBS with pH 6.8 with 100 μ L blood serum d) Cyclic voltammograms of 20 ppb ceftibuten in PBS with pH 6.8 with 100 μ L blood serum at different time interval i.e. initial, after 3 , 5 , 10 and 24 hours.

The voltammograms (Figure 3. 19) reveal distinct current signal peaks in blood serum at various potentials, yet no interference is observed at the potential corresponding to ceftibuten + 0.830 V. The current responses obtained from different concentrations of ceftibuten shows similar accuracy of method observed during the study in phosphate buffer pH 6.8 in absence of blood serum which confirms there is no interference from blood serum. From Figure 3. 19 (d) observed that, across different time intervals, the peak current response remains nearly unchanged (Table 3.11).

Table 3.11: Peak current response from cyclic voltammograms with time

Volunteer	Potential (V)	Current (μ A)					Difference in Current from T ₀ to T ₂₄ (μ A)	Remarks
		T ₀	T ₃	T ₅	T ₁₀	T ₂₄		
Volunteer-3	+0.8307	7.24	7.23	7.24	7.22	7.23	-0.02	Response remains almost consistent

The data shows the stability of both solution and modified electrode over 24 hours.

3.2.4.6 Study of ceftibuten and blood sample collected from human volunteer-3 by DPV using PGA modified GCE

Differential pulse voltammetric study of ceftibuten and blood serum was conducted using PGA-modified glassy carbon electrode in phosphate buffer solution pH 6.8 within the potential window of +0.1 V to +1.3 V at 50 mV/s scan rate. Measurement of blank was acquired from 10 mL of phosphate buffer solution pH 6.8. Then, ran DPV scan by adding 100 μ L blood serum. After that, added different concentrations of ceftibuten within it and acquired DPV. Also, acquired DPV measurements up to 24 hours at a regular interval.

The voltammograms (Figure 3. 20) reveal distinct current signal from blood serum at various potentials and no interference is observed at the same potential corresponding to ceftibuten + 0.883 V. The current responses obtained from different concentrations of ceftibuten shows similar accuracy of method observed during the study in phosphate buffer pH 6.8 in absence of blood serum which confirms there is no interference from blood serum.

From Figure 3. 20 (d) observed that, across different time intervals, the peak current response remains nearly constant.

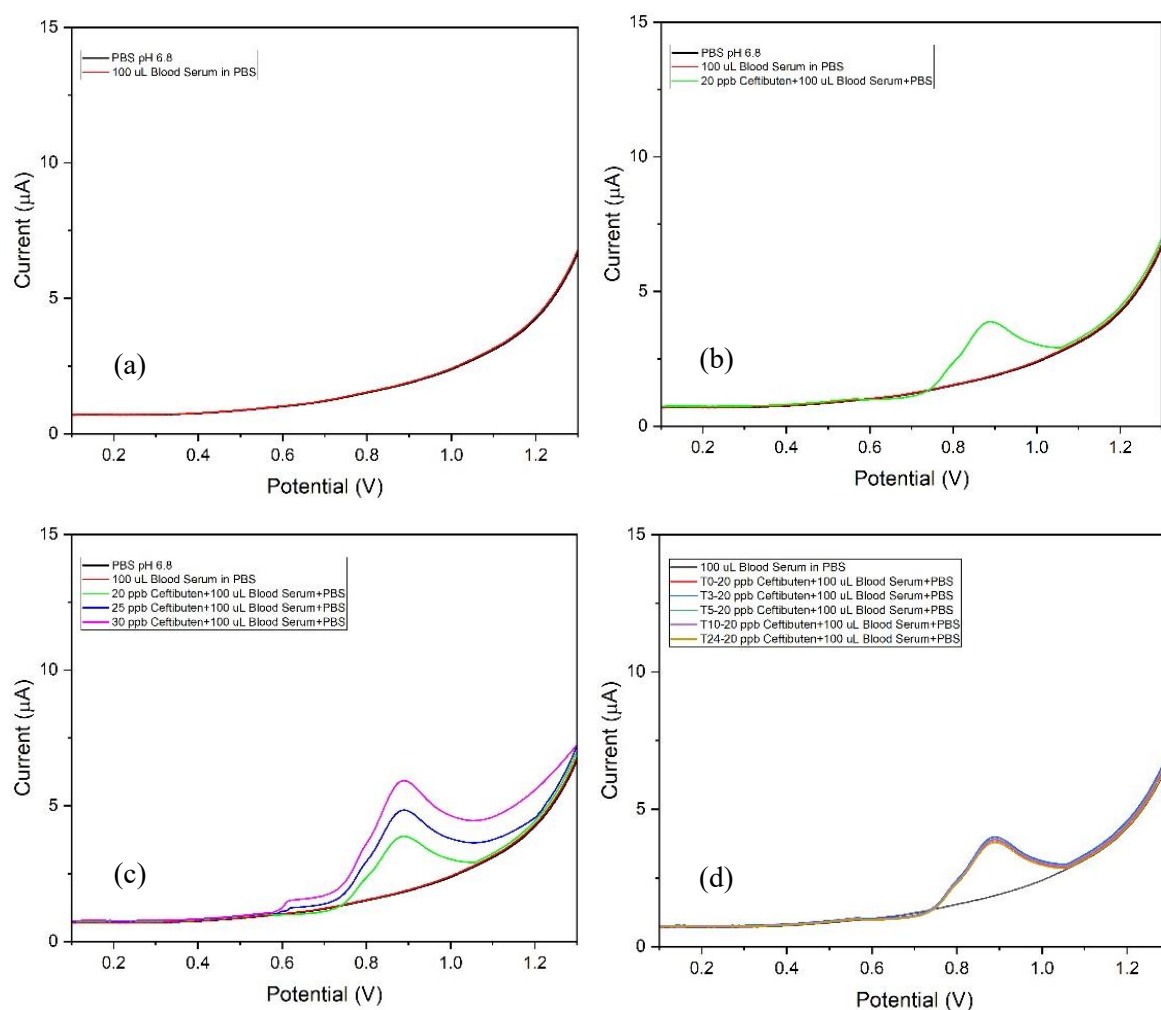


Figure 3. 20: Electrochemical responses recorded from blank, blood serum and different concentration of cefibuten.

a) Voltammograms of PBS with pH 6.8 as blank and 100 μL blood serum in PBS with pH 6.8 b) Voltammograms of 20 ppb cefibuten in PBS with pH 6.8 with 100 μL blood serum c) Voltammograms of 20, 25 and 30 ppb cefibuten in PBS with pH 6.8 with 100 μL blood serum d) Voltammograms of 20 ppb cefibuten in PBS with pH 6.8 with 100 μL blood serum at different time interval i.e. initial, after 3, 5, 10 hours and up to 24 hours.

Table 3.12: Peak current response from differential pulse voltammograms with time

Volunteer	Potential (V)	Current (μA)					Difference in Current from T_0 to T_{24} (μA)	Remarks
		T_0	T_3	T_5	T_{10}	T_{24}		
Volunteer-3	+0.883	3.914	3.914	3.913	3.914	3.914	-0.000	Response remains almost consistent

The data Table 3.12 shows the stability of both solution and modified electrode over 24 hours.

3.2.4.7 Summary of data of all real (blood) samples

From the voltammograms obtained from both cyclic voltammetry (CV) and differential pulse voltammetry (DPV), potential and peak currents are tabulated below and inferred as below-

Table 3.13: Evaluation of data obtained from cyclic voltammetric studies

Volunteer	Potential (V)	Current (μA)					Difference in Current from T_0 to T_{24} (μA)	Remarks
		T_0	T_3	T_5	T_{10}	T_{24}		
Volunteer-1	+0.8307	6.71	6.72	6.71	6.70	6.68	-0.03	Stable
Volunteer-2	+0.8401	8.09	8.08	8.09	8.07	8.06	-0.03	Stable
Volunteer-3	+0.8307	7.24	7.23	7.24	7.22	7.23	-0.02	Stable

Table 3.14: Evaluation of data obtained from differential pulse voltammetric studies

Volunteer	Potential (V)	Current (μA)					Difference in Current from T_0 to T_{24} (μA)	Remarks
		T_0	T_3	T_5	T_{10}	T_{24}		
Volunteer-1	+0.888	3.952	3.952	3.951	3.952	3.951	-0.001	Stable
Volunteer-2	+0.888	3.914	3.915	3.914	3.915	3.915	+0.001	Stable
Volunteer-3	+0.883	3.914	3.914	3.913	3.914	3.914	-0.000	Stable

The above data (Table 3.13 and 3.14) show, ceftibuten was remain almost constant over 24 hours which confirms it is stable up to 24 hours in such condition having phosphate buffer pH 6.8 and blood serum. The modified electrode can be used more than 24 hours. It also indicates, there was no further growth of bacteria happened during that period as well as there were no resistant bacteria which could degrade the ceftibuten. As a part of challenge study of above concurrence, in the next section conducted another study by incorporating β -lactamase enzyme within solution and measured the impact over a period of time.

3.2.5 In vitro study of antibiotic resistance against ceftibuten using β -lactamase enzyme

Based on the mechanism of action of ceftibuten (a β -lactam cephalosporin antibiotic), if any bacteria are susceptible to ceftibuten, it will bind to penicillin-binding proteins (PBPs) in the bacterial cell wall. This inhibits the synthesis of the bacterial cell wall, leading to cell lysis and ultimate death of those bacteria. The bacteria will be effectively killed or their growth will be inhibited³¹.

On the other hand, if the bacteria are resistant to ceftibuten, it may survive and continue to grow due to the following resistance mechanisms. Some bacteria can produce enzymes called

β -lactamases, which can break down the β -lactam ring in ceftibuten like cephalosporin or penicillin antibiotic, rendering it ineffective. If the bacteria produce extended-spectrum β -lactamases (ESBLs) or other β -lactamases that target ceftibuten, the antibiotic will be inactivated¹¹⁶. So, in this study β -Lactamase was added to see the impact. Blood sample was collected from the volunteer who is a resident of Muhsin Hall, Dhaka University. His blood group is B (+) ve and age was 25 years. The study of ceftibuten, blood serum and β -lactamase were conducted using PGA-modified glassy carbon electrode in phosphate buffer solution pH 6.8 by CV and DPV.

3.2.5.1 Study of ceftibuten with blood serum and β -lactamase enzyme by CV technique using PGA modified GCE

The CV analysis was conducted within a potential window of +0.1 V to +1.2 V at 50 mV/s scan rate. A blank CV run was recorded from 10 mL of phosphate buffer pH 6.8 solution. Then, ran CV scan by adding 300 μ L blood serum within it. Afterwards, added 10 ppb of ceftibuten within it and acquired CV. Finally, added 40 μ L β -lactamase considering above MIC within it and acquired CV for a period of time till got peak current for ceftibuten. The observation is captured in below Figure 3.21.

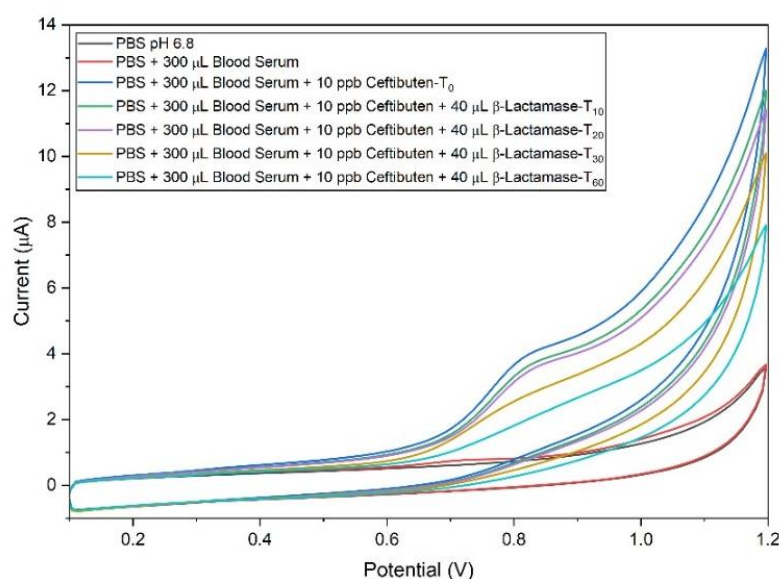


Figure 3. 21: Electrochemical responses recorded from PBS with pH 6.8, blood serum, ceftibuten before and after adding β -Lactamase

a) Voltammogram of PBS with pH 6.8 as blank b) Voltammogram of 300 μ L blood serum in PBS with pH 6.8 c) Voltammogram of 10 ppb ceftibuten in PBS with pH 6.8 with 300 μ L blood serum d) Voltammograms of 10 ppb ceftibuten in PBS with pH 6.8 with 300 μ L blood serum and 10 minutes, 20 minutes, 30 minutes and 60 minutes later after adding 40 μ L β -Lactamase.

Table 3.15: Data obtained from cyclic voltammetric study

Time	Potential (V)	Peak Current (μA)
Initial (before adding β -Lactamase)	+0.84	4.18
After adding 40 μL β -Lactamase-after 10 minutes	+0.84	3.84
After adding 40 μL β -Lactamase-after 20 minutes	+0.84	3.68
After adding 40 μL β -Lactamase-after 30 minutes	+0.84	3.04
After adding 40 μL β -Lactamase-after 60 minutes	+0.84	2.30

Based on peak response (Table 3.15), initially current response found above 4 μA and after adding β -lactamase it degraded significantly with respect to the time and after 60 minutes almost there was no response observed from ceftibuten. So, considering the data, as initially current response found above 4 μA and after adding β -lactamase it degraded significantly and after 60 minutes almost there was no response indicates sensitivity of ceftibuten.

3.2.5.2 Study of ceftibuten with blood serum and β -lactamase enzyme by DPV technique using PGA modified GCE

Like CV, DPV analysis was conducted within a potential window of +0.0 V to +1.3 V at 50 mV/s scan rate. A blank DPV run was recorded from 10 mL of phosphate buffer pH 6.8 solution. Then, scan by adding 300 μL blood serum within it.

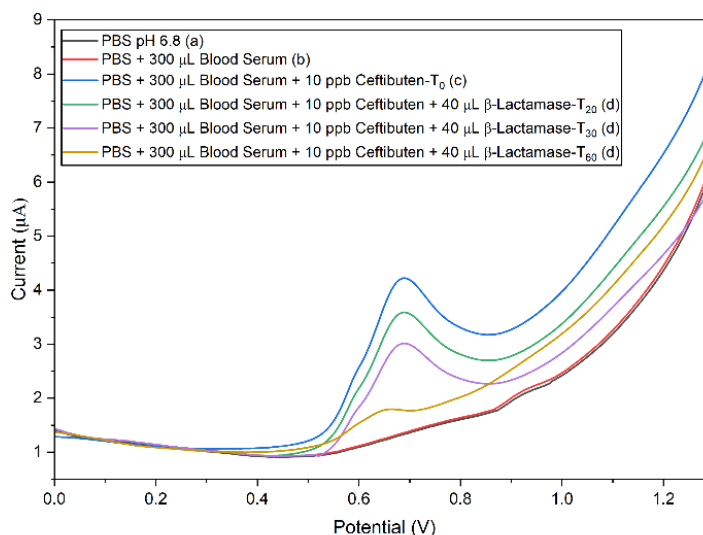


Figure 3. 22: Electrochemical responses recorded from PBS with pH 6.8, blood serum, ceftibuten before and after adding β -Lactamase

a) Voltammogram of PBS with pH 6.8 as blank b) Voltammogram of 300 μL blood serum in PBS with pH 6.8 c) Voltammogram of 10 ppb ceftibuten in PBS with pH 6.8 with 300 μL blood serum d) Voltammograms of 10 ppb ceftibuten in PBS with pH 6.8 with 300 μL blood serum and 10 minutes, 20 minutes, 30 minutes and 60 minutes later after adding 40 μL β -Lactamase.

Afterwards, added 10 ppb of ceftibuten within it and acquired DPV. Finally, added 40 μL β -Lactamase within it and acquired DPV for a period of time till got peak current for ceftibuten. The observation is captured in the above Figure 3.22.

Table 3.16: Data obtained from differential pulse voltammetric study

Time	Potential (V)	Peak Current (μA)
Initial (before adding β -Lactamase)	+0.68	4.21
After adding 40 μL β -Lactamase-after 20 minutes	+0.68	3.57
After adding 40 μL β -Lactamase-after 30 minutes	+0.68	3.01
After adding 40 μL β -Lactamase-after 60 minutes	+0.65	1.79

Based on peak response (Table 3.16), initially current response found above 4 μA and after adding β -Lactamase it degraded significantly with respect to the time and after 60 minutes almost there was no response observed from ceftibuten.

So, the theoretical assumption by which existing microbiological methods are being used to determine the susceptibility of microorganism can be determined by electrochemical techniques as well. Here the advantage is it can give some indication within few hours.

3.3 Study of Cefuroxime

Cefuroxime is classified as a second-generation cephalosporin antibiotic, characterized by the presence of a β -lactam ring a structural feature common to all members of the cephalosporin class. It is employed in both the treatment and prevention of various bacterial infections, including pneumonia, meningitis, otitis media, sepsis, urinary tract infections, and lyme disease¹⁵⁵. The molecular structure of cefuroxime is depicted in Figure 3.23-

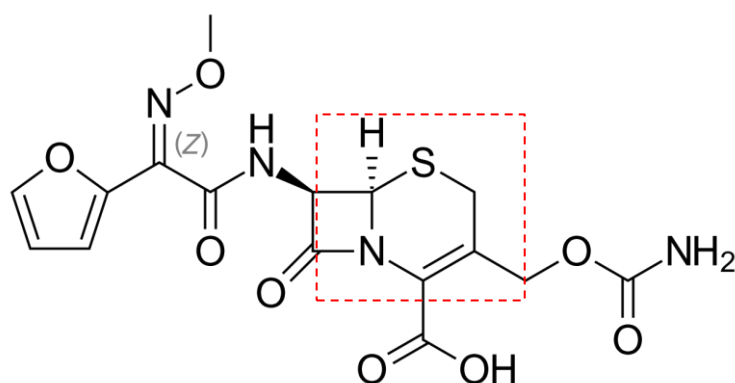


Figure 3. 23: Chemical structure of cefuroxime

Considering the objective of determination of antibiotic resistance, needed to develop a suitable and easy handling electrochemical methods which can be applied for cefuroxime

assay determination in different conditions. Specifically, the method should be capable to determine the content of cefuroxime in pure state and similarly when spiked with the blood sample. Accordingly, we applied CV, LSV and DPV considering the availability among various techniques.

In this study, from section 3.3.1 to section 3.3.3 captured the detailed explanation of the procedure of method development on assay or content determination of cefuroxime in pure state. In the next section 3.3.4, it has the details about the study of cefuroxime when spiked with blood sample as well as the inference of antibiotics behavior whether sensitive or resistant. Finally in section 3.3.5, explained the study outcome of in vitro study of antibiotic resistance using β -Lactamase enzyme to see the degradation pattern after adding within the solution of cefuroxime.

3.3.1 Cyclic voltammetric study of cefuroxime

The cyclic voltammetry study was carried out using PGA modified GCE in phosphate buffer solution at different pH from 4.5 to 7.4 within the potential window from -0.2 V to $+1.5$ V at various scan rates and various concentrations. The solubility of cefuroxime was checked in different pH of phosphate buffer and found it soluble at acidic, basic and neutral pH of phosphate buffer solution. Stock solution was prepared in phosphate buffer solution.

For the electrochemical set up, PGA modified GCE was used as working electrode, Ag/AgCl was used as reference electrode and platinum electrode was used as counter electrode and the potential window maintained from -0.2 V to $+1.5$ V.

3.3.1.1 Study of redox behavior of cefuroxime in phosphate buffer at wide pH range

To observe the redox behavior of cefuroxime, a series of cyclic voltammograms were taken with 20 ppb cefuroxime at 50 mV/s scan rate within the switching potential -0.2 V to $+1.5$ V vs. Ag/AgCl, which are shown in Figure 3.24. Peak current and pH variation are tabulated in Table 3.17.

Cyclic voltammograms were taken from 20 ppb cefuroxime solution within a potential range of -0.2 V to 1.5 V, a glassy carbon electrode modified with poly glutamic acid was used as working electrode. A platinum (Pt) wire was used as the counter electrode and an Ag/AgCl electrode was used as the reference electrode.

From Figure 3.24, we can see that, there is a single anodic peak found for each of the voltammogram between $+0.7$ V to $+0.9$ V which is oxidation peak could be happened due to

the oxidation of the β -lactam ring or sulfur-containing side chains. And, an irreversible reaction happened during this electrochemical process.

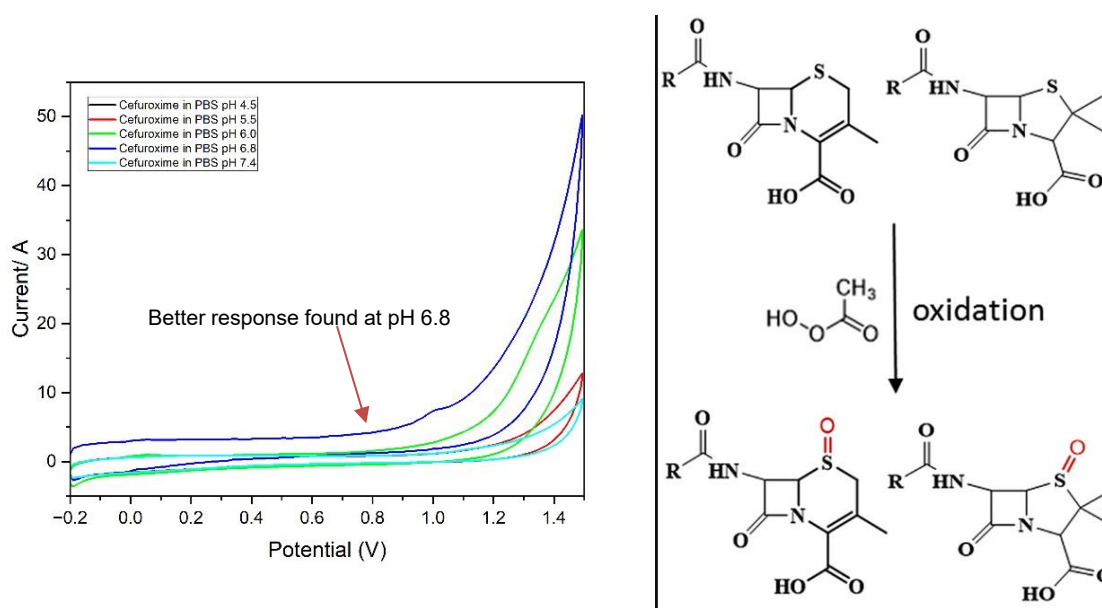


Figure 3. 24: Electrochemical responses and reactions happened during CV run.

- a) Cyclic voltammograms of 20 ppb cefuroxime in phosphate buffer at different pH using PGA modified GCE b) Electrochemical reaction happened in β -lactam ring

Based on the peak response observed from the voltammograms, no response found in most of the pH other than pH 6.8. From the voltammograms, better response found at pH 6.8 and the peak current found 7.58 μ A.

Table 3.17: Peak current response with the variation of pH for the response of 20 ppb cefuroxime in PBS at 50 mV/s scan rate

pH	Peak Current (μ A)
4.5	0
5.5	0
6.8	7.58
7.4	0

3.3.1.2 Study of minimum detection level of cefuroxime using PGA modified GCE

After selecting the suitable pH of phosphate buffer, studied the minimum detection level of cefuroxime using PGA modified GCE.

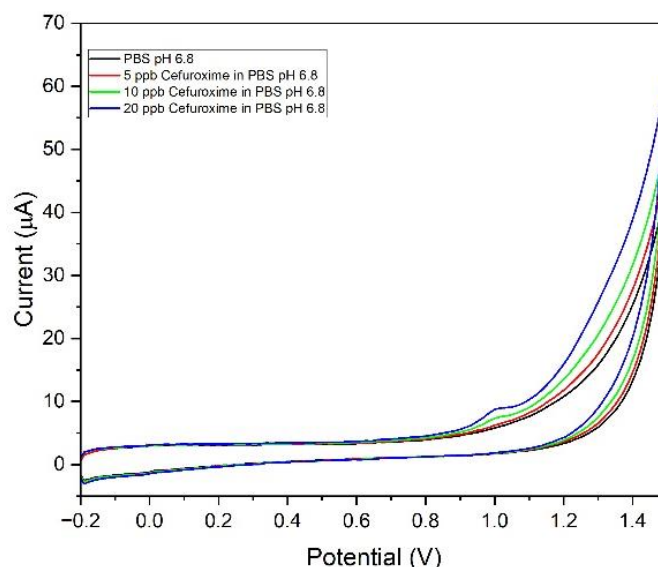


Figure 3. 25: Cyclic voltammograms of different concentrations of cefuroxime in PBS with pH 6.8 using PGA modified GCE at 50 mV/s scan rate

For this purpose, CV were taken for 5 ppb, 10 ppb and 20 ppb of cefuroxime solutions in PBS at pH 6.8. The voltammograms are overlaid and shown in Figure 3.25. Based on the data obtained from voltammograms, prominent peak response is observed from 10 ppb cefuroxime solution and very low response from 5 ppb solution. So, assumed it would be capable to detect above 5 ppb of cefuroxime if present in chemical or biological sample like blood sample.

3.3.1.3 Accuracy check of CV method for cefuroxime determination

Cyclic voltammograms (Figure-3.26) were taken from 5 ppb, 10 ppb and 20 ppb solutions of cefuroxime in PBS with pH 6.8 using PGA modified glassy carbon electrode.

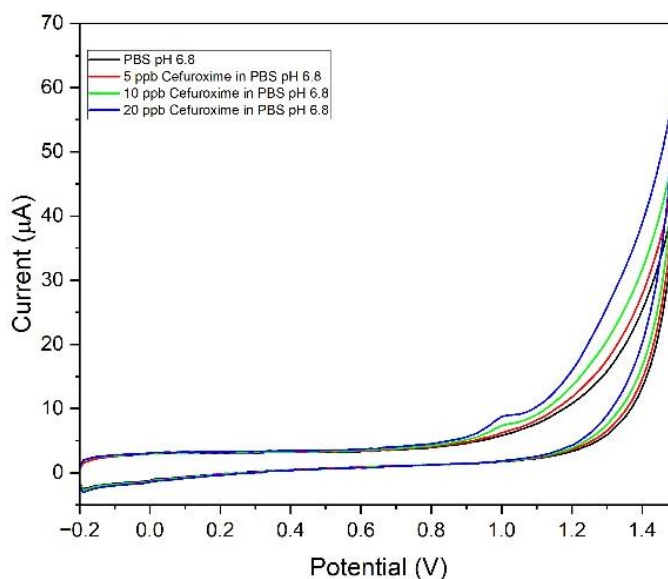


Figure 3. 26: Electrochemical responses (CV) of 5 ppb, 10 ppb and 20 ppb solutions of cefuroxime in PBS with pH 6.8 at 50 mV/s scan rate.

Data obtained from the voltammograms are captured in Table-3.18 and plotted graph in Figure 3.27. From above linearity curve, correlation coefficient found above 0.984 which shows a satisfactory accuracy and capability of detection. The peak potential has shifted slightly towards the anodic position with the increasing of the concentration of cefuroxime indicating irreversible charge transfer of cefuroxime.

Table 3.18: Electrochemical responses (peak current) of different concentration of cefuroxime at 50 mV/s scan rate

Concentration of Cefuroxime (ppb)	Peak Current (μA)
5	6.24
10	7.35
20	8.74

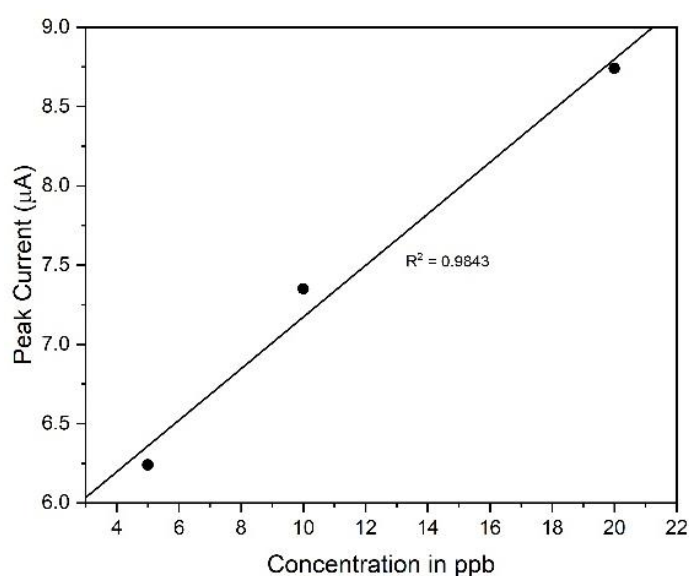


Figure 3. 27: Curve is showing linearity of CV method based on different concentration of cefuroxime.

3.3.1.4 Effect of scan rate variation on cefuroxime

Cyclic voltammograms were taken from 20 ppb cefuroxime in phosphate buffer solution pH 6.8 using PGA modified glassy carbon electrode by varying scan rate 25, 50, 75 and 100 mV/s within the potential window -0.2 V to $+1.5\text{ V}$. Cyclic voltammograms are shown in Figure 3.28 and peak current with scan rate variation are tabulated in Table 3.19.

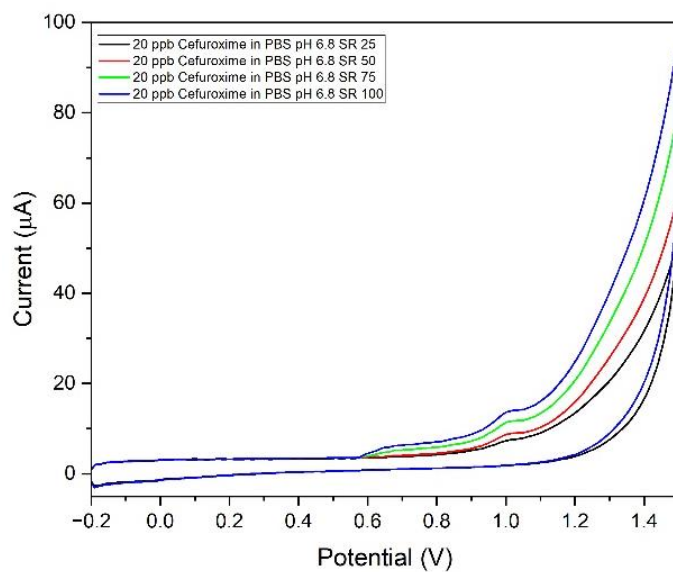


Figure 3. 28: Cyclic voltammograms of 20 ppb cefuroxime in PBS with pH 6.8 at 25, 50, 75 and 100 mV/s scan rate

Table 3.19: Electrochemical responses with scan rate variation from 20 ppb cefuroxime in PBS with pH 6.8

Scan Rate (v)/mV/s	$\sqrt{v}$	Peak Current (μ A)
25	5	7.25
50	7.07107	8.99
75	8.66025	11.36
100	10	13.50

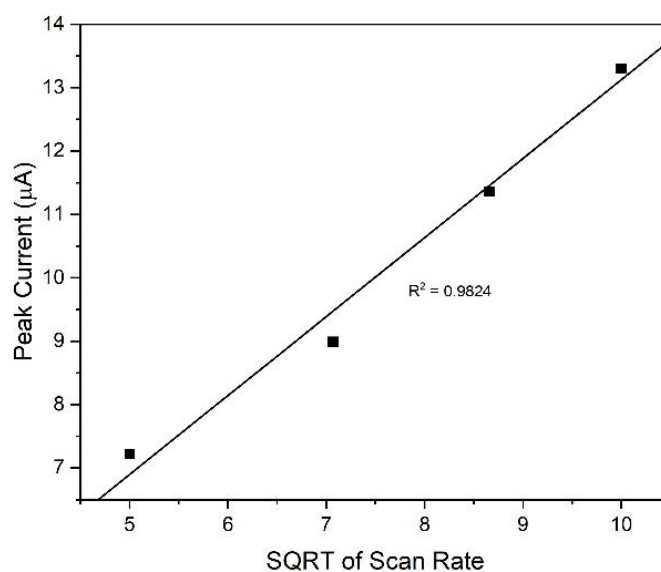


Figure 3. 29: Variation of peak current with SQRT of scan rate

The peak current data obtained from the scan rate variation are tabulated in Table-3.19. The calibration plot (Figure-3.29) shows a strong correlation coefficient value which indicates the robustness of the CV test method for cefuroxime determination.

So, based on the above all studies data it can be inferred, PGA modified glassy carbon electrode can determine the cefuroxime reproducibly using CV in phosphate buffer solution pH 6.8.

3.3.2 Study of cefuroxime by linear sweep voltammetry (LSV) technique

A linear sweep voltammetric analysis was conducted utilizing a PGA-modified glassy carbon electrode (GCE) within a phosphate buffer solution (PBS) at pH 6.8. The potential range was maintained between +0.2 V and +1.4 V, with variations in scan rates and concentrations. A stock solution was prepared in phosphate buffer solution, and subsequent experiments were performed under identical conditions, adjusting the concentration within the same potential window.

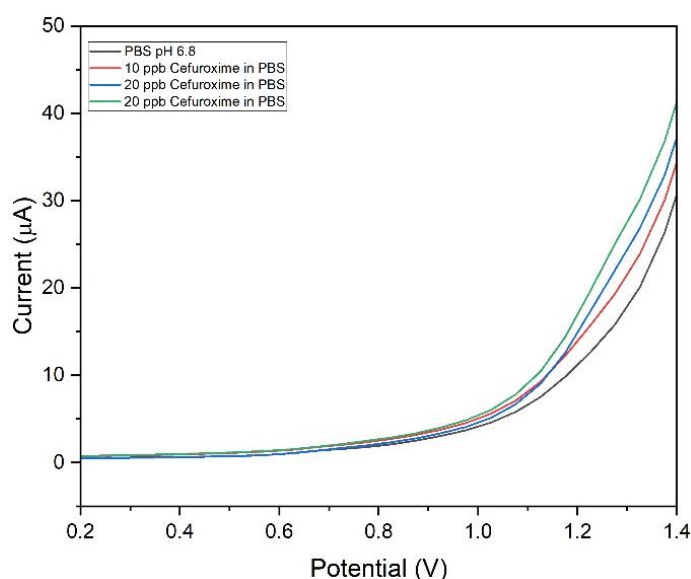


Figure 3. 30: Electrochemical responses (LSV) of 10 ppb, 20 ppb and 30 ppb solutions of cefuroxime in PBS with pH 6.8 at 50 mV/s scan rate

From the voltammograms (Figure 3.30) it has been observed that, PGA modified GCE is not showing distinctive response in LSV technique. However, the accuracy of LSV technique is not prominent as like Cyclic Voltammetry.

3.3.3 Differential pulse voltammetric study of cefuroxime

Differential pulse voltammetric study of cefuroxime was conducted using a PGA-modified

glassy carbon electrode in phosphate buffer solution pH 6.8. The electrochemical analysis was performed within a potential window of +0.4 V to +1.5 V, changing both the scan rates and analyte concentrations. The stock solution of cefuroxime was prepared in PBS with pH 6.8, and all experiments were carried out under identical pH conditions of 6.8.

From the voltammograms of Figure 3.31 it has been observed that, different concentrations of cefuroxime solutions show prominent responses with PGA modified GCE at 50 mV/s scan rate. This indicates a hope to detect a trace level of cefuroxime in real sample (Blood sample) which will help later to determine antibiotic resistance as well.

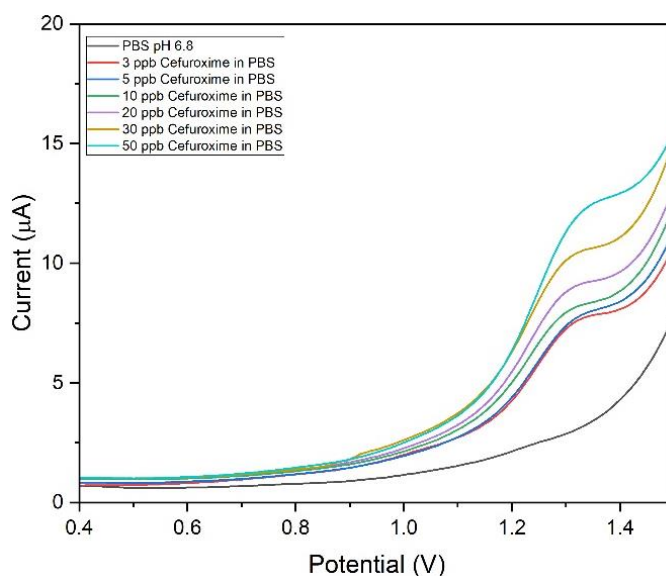


Figure 3. 31: Differential pulse voltammograms of 3 ppb, 5 ppb, 10 ppb, 20 ppb, 30 ppb and 50 ppb solutions of cefuroxime in PBS with pH 6.8 at 50 mV/s scan rate

3.3.4 Recovery study of cefuroxime in blood samples

The study was designed in such a way so that method recovery capabilities can be evaluated. For that, 10 mL phosphate buffer pH 6.8 was taken in a cell. Blank run has been captured with CV and DPV technique using PGA modified GCE at 50 mV/s scan rate. After that, added 100 µL blood serum within phosphate buffer solution and took voltammograms at CV and DPV using PGA modified GCE. Then, added different concentrations of cefuroxime solutions within above mentioned phosphate buffer and blood serum and, took voltammograms at CV and DPV using PGA modified GCE. Finally, solution having 20 ppb cefuroxime kept for more than 24 hours in room temperature around 35°C to 37°C and took voltammograms at CV and DPV using PGA modified GCE at a regular interval up to 24 hours. Blood samples were collected from volunteers, along with relevant medical history and physical conditions data essential to the study.

3.3.4.1 Study of cefuroxime and blood sample collected from human volunteer-1 by CV using PGA modified GCE

To collect blood serum, centrifuged blood samples collected from human volunteers. In this study, the volunteers blood group is A (+) ve. His body weight was 78 kg and age was 26 years. The volunteer was the resident of Mirpur, Dhaka.

Cyclic voltammetric study of cefuroxime and blood serum was conducted using PGA-modified glassy carbon electrode in phosphate buffer solution pH 6.8. The electrochemical analysis was performed within a potential window of -0.2 V to $+1.5$ V at 50 mV/s scan rate. Blank CV run was recorded from 10 mL of phosphate buffer pH 6.8 solution. Then, ran CV scan by adding 100 μ L blood serum. Afterwards, added different concentrations of cefuroxime within it and acquired CV. Also, acquired CV measurements up to 24 hours at a regular interval.

The voltammograms reveal distinct current signal peaks in blood serum at various potentials, yet no interference is observed at the potential corresponding to cefuroxime $+1.000$ V. The current responses obtained from different concentrations of cefuroxime shows similar accuracy of method observed during the study in phosphate buffer pH 6.8 in absence of blood serum which confirms there is no interference from blood serum.

From Figure 3. 32 (d), it has been observed that, across the different time intervals, the peak current response was remained almost unchanged.

Table 3.20: Peak current response from cyclic voltammograms with time

Volunteer	Potentia	Current (μ A)					Difference in Current from T_0 to T_{24} (μ A)	Remarks
	I (V)	T_0	T_3	T_5	T_{10}	T_{24}		
Volunteer-1	$+1.000$	8.817	8.812	8.8213	8.816	8.811	-0.006	Response remains almost consistent

The data (Table 3.20) shows the stability of both solution and modified electrode over 24 hours. It also indicates, subject volunteer blood doesn't have any bacteria as well as resistant bacteria which can generates β -Lactamase. So, peak current remains almost consistent of cefuroxime observed over a period of time.

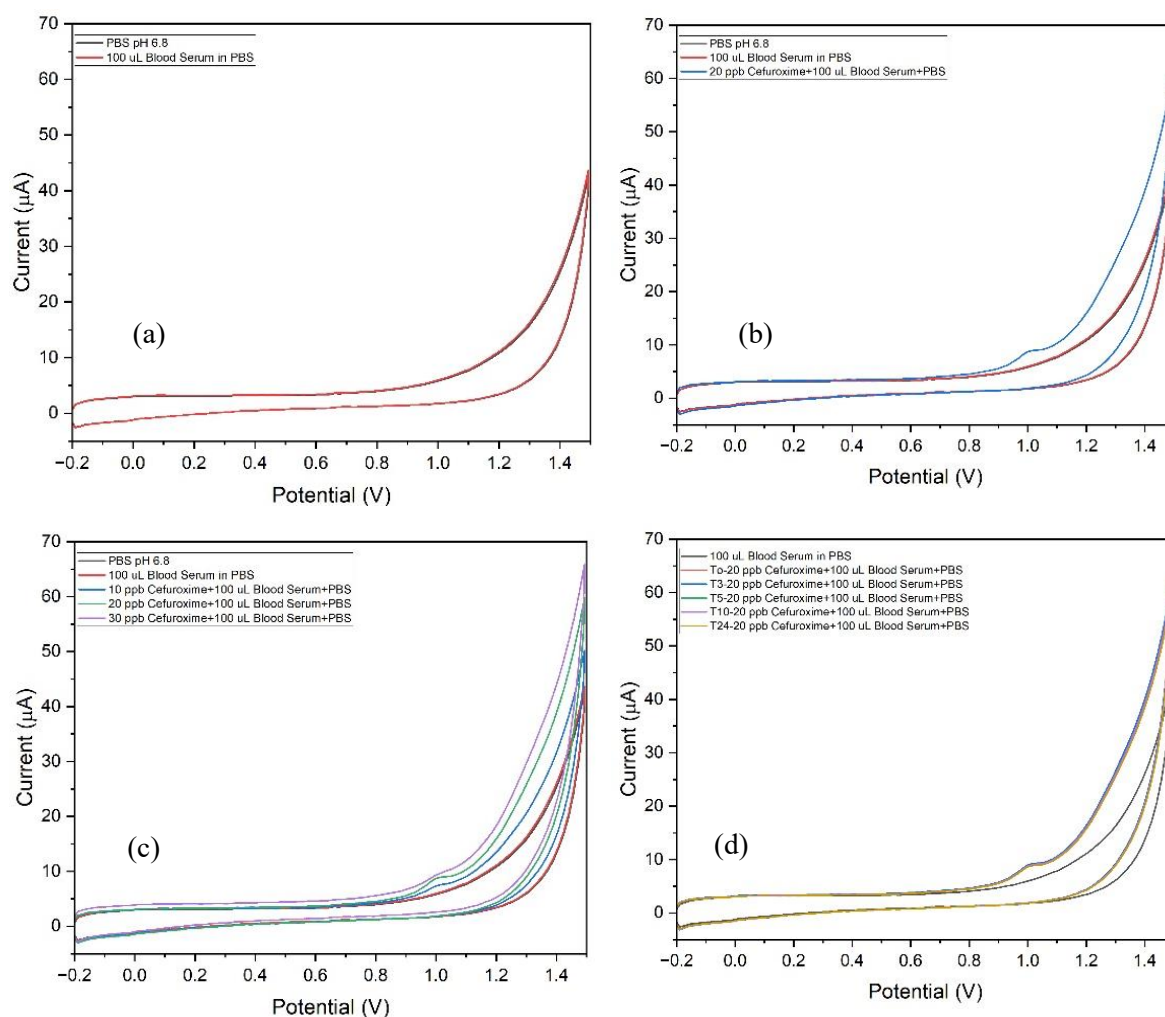


Figure 3. 32: Electrochemical responses recorded from blank, blood serum and different concentration of cefuroxime.

a) Cyclic voltammograms of PBS with pH 6.8 as blank and 100 μ L blood serum in PBS with pH 6.8 b) Cyclic voltammograms of 20 ppb cefuroxime in PBS with pH 6.8 with 100 μ L blood serum c) Cyclic voltammograms of 10, 20 and 30 ppb cefuroxime in PBS with pH 6.8 with 100 μ L blood serum d) Cyclic voltammograms of 20 ppb cefuroxime in PBS with pH 6.8 with 100 μ L blood serum at different time interval i.e. initial, after 3, 5, 10 and 24 hours.

3.3.4.2 Study of cefuroxime and blood sample collected from human volunteer-1 by DPV using PGA modified GCE

Differential pulse voltammetric study of cefuroxime and blood serum was conducted using PGA-modified glassy carbon electrode in phosphate buffer solution pH 6.8 within the potential window of +0.4 V to +1.6 V at 50 mV/s scan rate. Measurement of blank was acquired from 10 mL of phosphate buffer solution pH 6.8. Then, ran DPV scan by adding 100 μ L blood serum. After that, added different concentrations of cefuroxime within it and

acquired voltammogram. Also, acquired DPV measurements up to 24 hours at a regular interval.

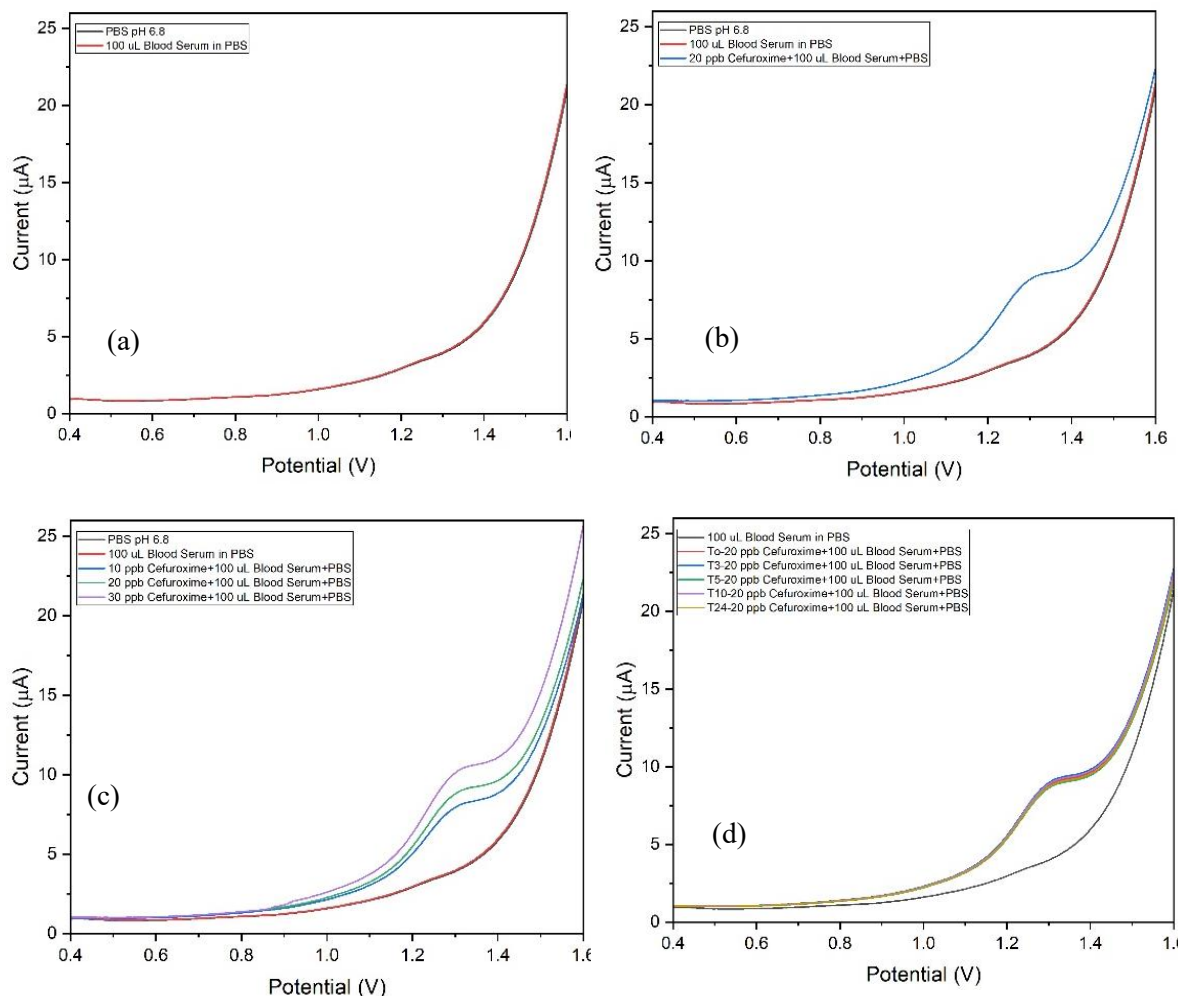


Figure 3. 33: Electrochemical responses recorded from blank, blood serum and different concentration of cefuroxime.

a) Voltammograms of PBS with pH 6.8 as blank and 100 μL blood serum in PBS with pH 6.8 b) Voltammograms of 20 ppb cefuroxime in PBS with pH 6.8 with 100 μL blood serum c) Voltammograms of 10, 20 and 30 ppb cefuroxime in PBS with pH 6.8 with 100 μL blood serum d) Voltammograms of 20 ppb cefuroxime in PBS with pH 6.8 with 100 μL blood serum at different time interval i.e. initial, after 3, 5, 10 hours and up to 24 hours.

The voltammograms reveal distinct current signal from blood serum at various potentials and no interference is observed at the same potential corresponding to cefuroxime + 1.316 V. The current responses obtained from different concentrations of cefuroxime shows similar accuracy of method observed during the study in phosphate buffer pH 6.8 in absence of blood serum which confirms there is no interference from blood serum.

Significantly, Figure 3.33 (d) shows that the peak current responses remain almost constant across different time intervals.

Table 3.21: Peak current response from differential pulse voltammograms with time

Volunteer	Potential (V)	Current (μA)					Difference in Current from T_0 to T_{24} (μA)	Remarks
		T_0	T_3	T_5	T_{10}	T_{24}		
Volunteer-1	+1.316	9.206	9.201	9.197	9.203	9.201	-0.005	Current response remains almost consistent

Based on the above data (Table 3.21), the subject volunteer blood doesn't have any resistant bacteria which can generate β -Lactamase. So, peak current remains almost consistent of cefuroxime observed over a period of time. Similarly, it is assumed, no further growth of microorganism happened after applying cefuroxime. So, no decrease of current response observed.

3.3.4.3 Study of cefuroxime and blood sample collected from human volunteer-2 by CV using PGA modified GCE

Blood sample was collected from the volunteer who is a resident of Hajaribag, Dhaka. His blood group is A (+) ve and body weight was 70 kg and age was 20 years old. The electrochemical investigation of cefuroxime in blood serum was performed using a PGA-modified glassy carbon electrode, with phosphate buffer solution (pH 6.8) as the supporting electrolyte. The measurements were carried out within a potential range of -0.2 V to $+1.5$ V at a scan rate of 50 mV/s. Initially, a blank cyclic voltammogram (CV) was recorded using 10 mL of the phosphate buffer solution. Subsequently, a CV scan was obtained after introducing 100 μL of blood serum. Different concentrations of cefuroxime were then incrementally added, and corresponding CV responses were recorded. Additionally, cyclic voltammetry measurements were taken at regular intervals over a 24-hour period to assess stability and time-dependent behavior.

From the voltammograms it has been observed that, there are some very low signals in blood serum at various potentials, but no interference is observed at the potential corresponding to cefuroxime $+ 1.001$ V. The current responses obtained from different concentrations of cefuroxime shows similar accuracy of method observed during the study in phosphate buffer pH 6.8 in absence of blood serum which confirms there is no interference from blood serum.

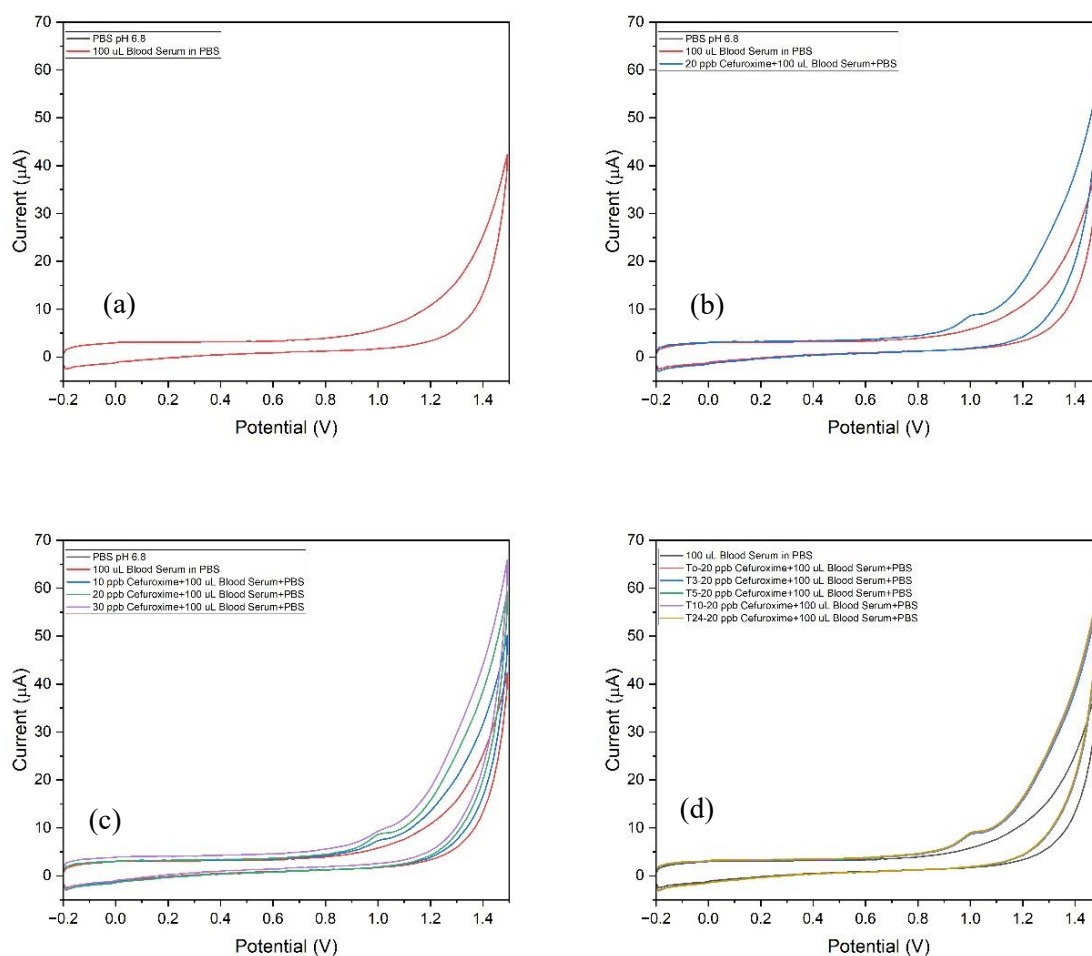


Figure 3. 34: Electrochemical responses recorded from blank, blood serum and different concentration of cefuroxime.

a) Cyclic voltammograms of PBS with pH 6.8 as blank and 100 μL blood serum in PBS with pH 6.8 b) Cyclic voltammograms of 20 ppb cefuroxime in PBS with pH 6.8 with 100 μL blood serum c) Cyclic voltammograms of 10, 20 and 30 ppb cefuroxime in PBS with pH 6.8 with 100 μL blood serum d) Cyclic voltammograms of 20 ppb cefuroxime in PBS with pH 6.8 with 100 μL blood serum at different time interval i.e. initial, after 3, 5, 10 and 24 hours.

Table 3.22: Peak current response from cyclic voltammograms with time

Volunteer	Potential (V)	Current (μA)					Difference in Current from T_0 to T_{24} (μA)	Remarks
		T_0	T_3	T_5	T_{10}	T_{24}		
Volunteer-2	+1.001	8.822	8.823	8.825	8.815	8.823	+0.001	Peak current remains almost consistent

As seen in Figure 3.34 (d), the peak current responses stay largely consistent over different time periods. The data (Table 3.22) shows the stability of both solution and modified electrode over 24 hours.

3.3.4.4 Study of cefuroxime and blood sample collected from human volunteer-2 by DPV using PGA modified GCE

The electrochemical behavior of cefuroxime in blood serum was investigated using a PGA-modified glassy carbon electrode in a phosphate buffer solution (pH 6.8). Differential pulse voltammetry (DPV) measurements were performed within a potential range of +0.4 V to +1.6 V at a scan rate of 50 mV/s. Initially, a blank DPV response was recorded using 10 mL of the phosphate buffer solution. Subsequently, 100 μ L of blood serum was introduced, and a DPV scan was obtained. Different concentrations of cefuroxime were then added sequentially, and corresponding DPV responses were recorded. Additionally, DPV measurements were conducted at regular intervals over a 24-hour period to evaluate stability and time-dependent effects.

The voltammograms (Figure 3. 35) reveal distinct current signal from blood serum at various potentials and no interference is observed at the same potential corresponding to cefuroxime + 1.315 V. The current responses obtained from different concentrations of cefuroxime shows similar accuracy of method observed during the study in phosphate buffer pH 6.8 in absence of blood serum which confirms there is no interference from blood serum.

Figure 3.35 (d) demonstrates that the peak current responses (Table 3.23) are nearly unaffected by changes in time intervals.

Table 3.23: Peak current response from differential pulse voltammograms with time

Volunteer	Potential (V)	Current (μ A)					Difference in Current from T ₀ to T ₂₄ (μ A)	Remarks
		T ₀	T ₃	T ₅	T ₁₀	T ₂₄		
Volunteer-2	+1.315	9.203	9.204	9.199	9.203	9.206	+0.003	Response remains almost consistent of cefuroxime over the period of time

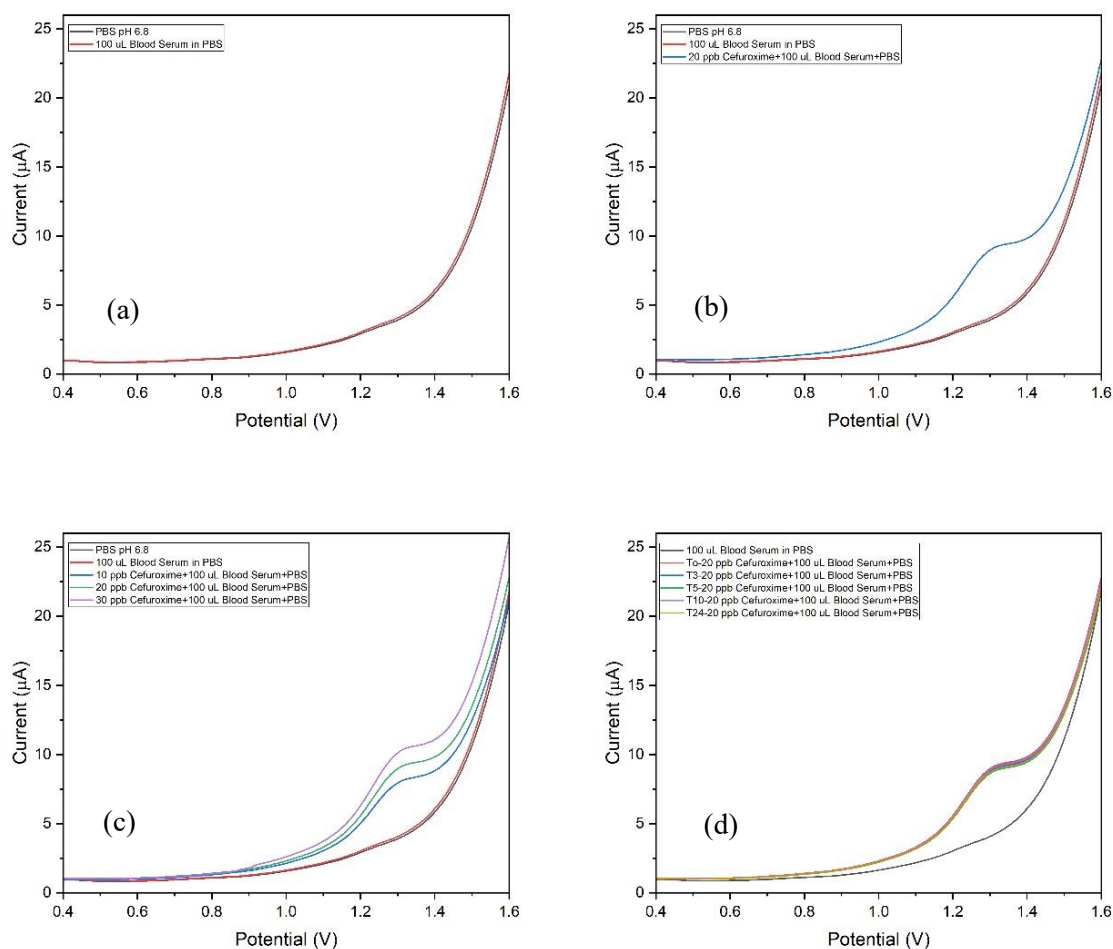


Figure 3. 35: Electrochemical responses recorded from blank, blood serum and different concentration of cefuroxime.

a) Voltammograms of PBS with pH 6.8 as blank and 100 μL blood serum in PBS with pH 6.8 b) Voltammograms of 20 ppb cefuroxime in PBS with pH 6.8 with 100 μL blood serum c) Voltammograms of 10, 20 and 30 ppb cefuroxime in PBS with pH 6.8 with 100 μL blood serum d) Voltammograms of 20 ppb cefuroxime in PBS with pH 6.8 with 100 μL blood serum at different time interval i.e. initial, after 3, 5, 10 hours and up to 24 hours.

3.3.4.5 Study of cefuroxime and blood sample collected from human volunteer-3 by CV using PGA modified GCE

As a part of study, collected blood samples from human volunteers. Then, centrifuged it to separate blood serum and used blood serum in study. In this study, the volunteers blood group was A (+) ve. His body weight was 64 kg and age was 22 years. The volunteer was the resident of Shahidullah Hall, Dhaka University.

The electrochemical behavior of cefuroxime in blood serum was investigated through cyclic voltammetry using a PGA-modified glassy carbon electrode. The experiments were carried out in phosphate buffer solution (pH 6.8) with an applied potential range of -0.2 V to +1.5 V at a scan rate of 50 mV/s. Initial measurements began with recording a blank cyclic voltammogram using 10 mL of the phosphate buffer solution. Subsequently, 100 μ L of blood serum was introduced into the system, followed by CV analysis. The study then proceeded with sequential additions of varying cefuroxime concentrations, with corresponding voltammograms recorded for each increment.

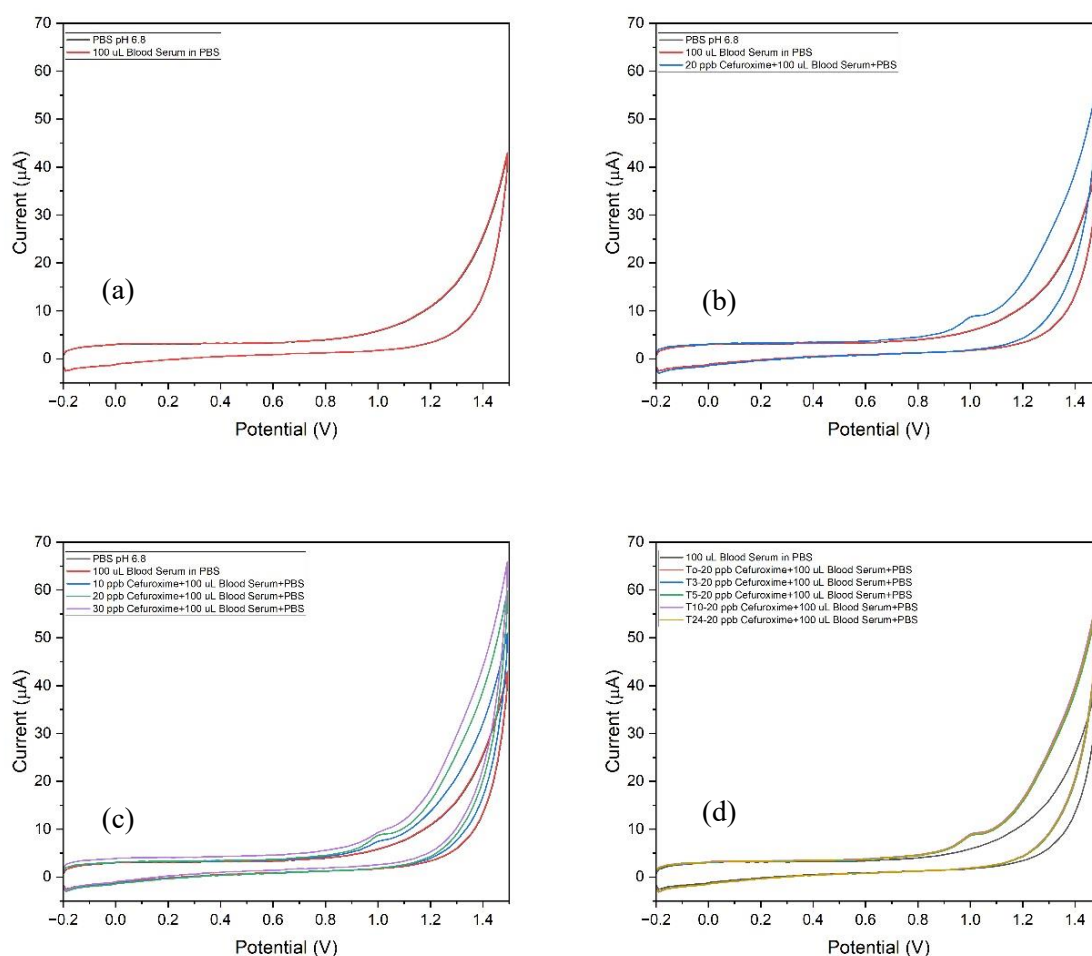


Figure 3. 36: Electrochemical responses recorded from blank, blood serum and different concentration of cefuroxime.

a) Cyclic voltammograms of PBS with pH 6.8 as blank and 100 μ L blood serum in PBS with pH 6.8 b) Cyclic voltammograms of 20 ppb cefuroxime in PBS with pH 6.8 with 100 μ L blood serum c) Cyclic voltammograms of 10, 20 and 30 ppb cefuroxime in PBS with pH 6.8 with 100 μ L blood serum d) Cyclic voltammograms of 20 ppb cefuroxime in PBS with pH 6.8 with 100 μ L blood serum at different time interval i.e. initial, after 3, 5, 10 and 24 hours.

To assess temporal stability, cyclic voltametric measurements were periodically collected over a 24-hour duration. The voltammograms reveal no distinct current signal peaks in blood serum at the same potential corresponding to cefuroxime + 1.002 V. The current responses obtained from different concentrations of cefuroxime shows similar accuracy of method observed during the study in phosphate buffer pH 6.8 in absence of blood serum which confirms there is no interference from blood serum. Noteworthy, the peak current responses in Figure 3.36 (d) exhibit minimal variation at different time intervals. The data (Table 3.24) shows the stability of both solution and modified electrode over 24 hours.

Table 3.24: Peak current response from cyclic voltammograms with time

Volunteer	Potential (V)	Current (μA)					Difference in Current from T_0 to T_{24} (μA)	Remarks
		T_0	T_3	T_5	T_{10}	T_{24}		
Volunteer-3	+1.002	8.827	8.823	8.824	8.825	8.824	-0.003	Current remains almost consistent

3.3.4.6 Study of cefuroxime and blood sample collected from human volunteer-3 by DPV using PGA modified GCE

Differential pulse voltammetric study of cefuroxime and blood serum was conducted using PGA-modified glassy carbon electrode in phosphate buffer solution pH 6.8 within the potential window of +0.4 V to +1.6 V at 50 mV/s scan rate. Measurement of blank was acquired from 10 mL of phosphate buffer solution pH 6.8. Then, ran DPV scan by adding 100 μL blood serum. After that, added different concentrations of cefuroxime within it and acquired DPV. Also, acquired DPV measurements up to 24 hours at a regular interval.

The voltammetric analysis demonstrates well-defined current signals for blood serum components across multiple potentials, while showing no interfering signals at the characteristic oxidation potential of cefuroxime (+1.316 V). Notably, the current responses for various cefuroxime concentrations exhibit comparable accuracy to those obtained in pure phosphate buffer (pH 6.8), confirming the absence of significant matrix interference from blood serum components.

As seen in Figure 3.37 (d), the peak current responses stay mostly consistent over different time periods.

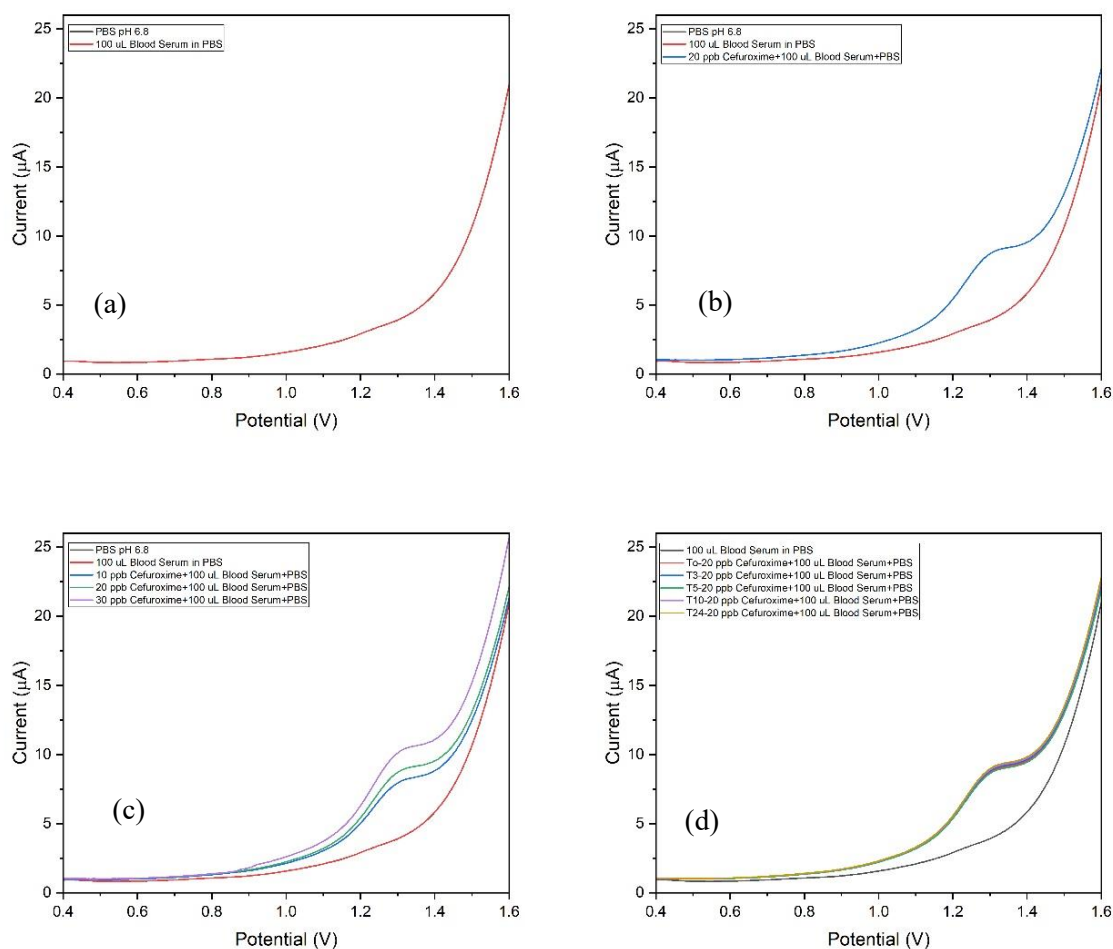


Figure 3. 37: Electrochemical responses recorded from blank, blood serum and different concentration of cefuroxime.

a) Voltammograms of PBS with pH 6.8 as blank and 100 μL blood serum in PBS with pH 6.8 b) Voltammograms of 20 ppb cefuroxime in PBS with pH 6.8 with 100 μL blood serum c) Voltammograms of 10, 20 and 30 ppb cefuroxime in PBS with pH 6.8 with 100 μL blood serum d) Voltammograms of 20 ppb cefuroxime in PBS with pH 6.8 with 100 μL blood serum at different time interval i.e. initial, after 3, 5, 10 hours and up to 24 hours.

Table 3.25: Peak current response from differential pulse voltammograms with time

Volunteer	Potential (V)	Current (μA)					Difference in Current from T_0 to T_{24} (μA)	Remarks
		T_0	T_3	T_5	T_{10}	T_{24}		
Volunteer-3	+1.316	9.201	9.205	9.197	9.203	9.195	-0.006	Peak response remains almost consistent

The data Table 3.25 shows the stability of both solution and modified electrode over 24 hours.

3.3.4.7 Summary of data of all real (blood) samples:

From the voltammograms obtained from both cyclic voltammetry (CV) and differential pulse voltammetry (DPV), potential and peak currents are tabulated below and inferred as below-

Table 3.26: Evaluation of data obtained from cyclic voltammetric study

Volunteer	Potential	Current (μA)					Difference in Current from T_0 to T_{24} (μA)	Remarks
	I (V)	T_0	T_3	T_5	T_{10}	T_{24}		
Volunteer-1	+1.000	8.817	8.812	8.8213	8.816	8.811	-0.006	Stable
Volunteer-2	+1.001	8.822	8.823	8.825	8.815	8.823	+0.001	Stable
Volunteer-3	+1.002	8.827	8.823	8.824	8.825	8.824	-0.003	Stable

Table 3.27: Evaluation of data obtained from differential pulse voltammetric study

Volunteer	Potential	Current (μA)					Difference in Current from T_0 to T_{24} (μA)	Remarks
	I (V)	T_0	T_3	T_5	T_{10}	T_{24}		
Volunteer-1	+1.316	9.206	9.201	9.197	9.203	9.201	-0.001	Stable
Volunteer-2	+1.315	9.203	9.204	9.199	9.203	9.206	+0.003	Stable
Volunteer-3	+1.316	9.201	9.205	9.197	9.203	9.195	-0.006	Stable

The above data (Table 3.26 and 3.27) show, cefuroxime was remained almost constant over 24 hours which confirms it is stable up to 24 hours in such condition having phosphate buffer pH 6.8 and blood serum. It also indicates, there was no further growth of bacteria happened during that period as well as there were no resistant bacteria which could degrade the cefuroxime. As a part of challenge study of above concurrence, conducted another study by incorporating β -Lactamase enzyme within solution and measured the impact over a period of time.

Cefuroxime is a beta-lactam antibiotic which works by binding to specific penicillin-binding proteins (PBPs) located inside the bacterial cell wall. It works by interfering with the formation of bacteria cell walls. It causes the cell walls to rupture (break) leading to the death of the bacteria. Based on the evaluation of mechanism of action of cefuroxime, If the bacteria are resistant to cefuroxime the bacteria may survive and continue to grow due to one or more of the following resistance mechanisms-

β -lactamase Production: Many resistant bacteria produce β -lactamase enzymes, which break down the beta-lactam ring in cefuroxime, rendering it ineffective. Some β -lactamases, such as TEM-1 or SHV-1, can hydrolyze cefuroxime, while others, like Extended-spectrum β -lactamases (ESBLs), may also target it. If the bacteria produce β -lactamases that can degrade cefuroxime, the antibiotic will be inactivated. So, it is assumed content of cefuroxime will degrade with respect to time even in in-vitro condition if the blood serum possess any resistance towards Cefuroxime.

3.3.5 In vitro study of antibiotic resistance against cefuroxime using β -lactamase enzyme

Based on the mechanism of action of cefuroxime, if any bacteria is susceptible to cefuroxime, it will bind to penicillin-binding proteins (PBPs) in the bacterial cell wall. This inhibits the synthesis of the bacterial cell wall, leading to cell lysis and ultimate death of those bacteria. The bacteria will effectively be killed or their growth would be inhibited³¹.

On the other hand, if the bacteria is resistant to cefuroxime, it may survive and continue to grow due to the following resistance mechanisms. Some bacteria produce enzymes called β -lactamases, which can break down the β -lactam ring present in cefuroxime like cephalosporin or penicillin antibiotic, rendering it ineffective. If the bacteria produce extended-spectrum β -lactamases (ESBLs) or other β -lactamases that target cefuroxime, the antibiotic will be inactivated¹¹⁶.

So, in this study β -Lactamase enzyme was added to see the impact. Blood sample was collected from the volunteer who is a resident of Bangamata Hall, Dhaka University. Her blood group is O (+) ve and body weight was 55 kg and age was 22 years old.

The study of cefuroxime, blood serum and β -Lactamase were conducted using PGA-modified glassy carbon electrode in phosphate buffer solution pH 6.8 by CV and DPV.

3.3.5.1 Study of cefuroxime with blood serum and β -lactamase enzyme by CV technique using PGA modified GCE

The CV analysis was conducted within a potential window of -0.2 V to $+1.5$ V at 50 mV/s scan rate. A blank CV run was recorded from 10 mL of phosphate buffer pH 6.8 solution. Then, ran CV scan by adding 300 μ L blood serum within it. Afterwards, added 10 ppb of cefuroxime within it and acquired voltammogram. Finally, added 40 μ L β -lactamase within it and acquired CV for a period of time till got peak current for cefuroxime. The observation is captured in below Figure 3.38.

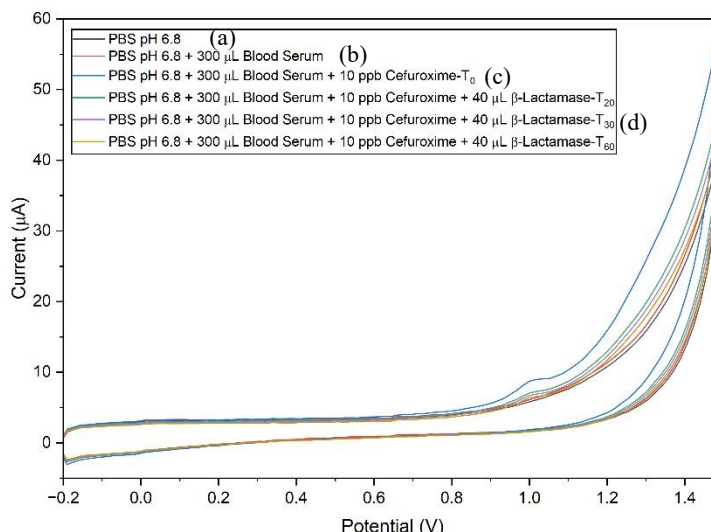


Figure 3. 38: Electrochemical responses recorded from PBS with pH 6.8, blood serum, cefuroxime before and after adding β -Lactamase.

a) Voltammogram of PBS with pH 6.8 as blank b) Voltammogram of 300 μ L blood serum in PBS with pH 6.8 c) Voltammogram of 10 ppb cefuroxime in PBS with pH 6.8 with 300 μ L blood serum d. Voltammograms of 10 ppb cefuroxime in PBS with pH 6.8 with 300 μ L blood serum and 10 minutes, 20 minutes, 30 minutes and 60 minutes later after adding 40 μ L β -Lactamase.

Table 3.28: Data obtained from cyclic voltammetric study

Time	Potential (V)	Peak Current (μ A)
Initial (before adding β -Lactamase)	+1.01	8.96
After adding 40 μ L β -Lactamase-after 20 minutes	+1.01	7.05
After adding 40 μ L β -Lactamase-after 30 minutes	+1.01	6.90
After adding 40 μ L β -Lactamase-after 60 minutes	+1.01	6.33

Based on peak response (Table 3.28), initially current response found above 8.9 μ A and after adding β -lactam it degraded significantly with respect to the time and after 60 minutes current response degraded like phosphate buffer blank and almost no response observed from cefuroxime.

3.3.5.2 Study of cefuroxime with blood serum and β -lactamase enzyme by DPV technique using PGA modified GCE

Similarly, DPV analysis was conducted within a potential window of +0.4 V to +1.6 V at 50 mV/s scan rate. A blank DPV run was recorded from 10 mL of phosphate buffer pH 6.8 solution. Then, scan by adding 300 μ L blood serum within it. Afterwards, added 10 ppb of cefuroxime within it and acquired DPV. Finally, added 40 μ L β -Lactamase within it and

acquired DPV for a period of time till getting peak current for cefuroxime. The observation is captured in below Figure 3. 39.

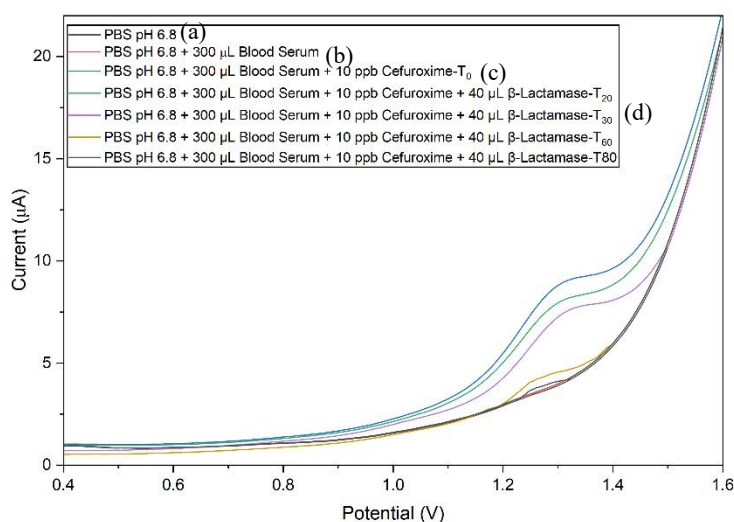


Figure 3. 39: Electrochemical responses recorded from PBS with pH 6.8, blood serum, cefuroxime before and after adding β -Lactamase

a) Voltammogram of PBS with pH 6.8 as blank b) Voltammogram of 300 μ L blood serum in PBS with pH 6.8 c) Voltammogram of 10 ppb cefuroxime in PBS with pH 6.8 with 300 μ L blood serum d) Voltammograms of 10 ppb cefuroxime in PBS with pH 6.8 with 300 μ L blood serum and 10 minutes, 20 minutes, 30 minutes and 60 minutes later after adding 40 μ L β -Lactamase.

Table 3.29: Data obtained from differential pulse voltammetric study

Time	Potential (V)	Peak Current (μ A)
Initial (before adding β -Lactamase)	+1.32	9.03
After adding 40 μ L β -Lactamase-after 20 minutes	+1.32	8.10
After adding 40 μ L β -Lactamase-after 30 minutes	+1.32	7.52
After adding 40 μ L β -Lactamase-after 60 minutes	+1.23	4.35
After adding 40 μ L β -Lactamase-after 80 minutes	+1.23	3.85

Based on peak response (Table 3.29), initially current response found above 9 μ A and after adding β -Lactamase it degraded significantly with respect to the time and after 80 minutes almost there was no response observed from cefuroxime. So, the theoretical assumption by which existing microbiological methods are being used to determine the susceptibility of microorganism can be determined by electrochemical techniques as well. Here the advantage is it can give some indication within 1 to 2 hours.

3.4 Study of Levofloxacin

Levofloxacin is a broad-spectrum antimicrobial agent belonging to the fluoroquinolone class of antibiotics. It demonstrates efficacy against a wide range of both gram positive and gram-negative bacterial pathogens. Its pharmacological action involves the inhibition of bacterial DNA gyrase and topoisomerase IV key enzymes required for DNA replication and transcription¹⁵⁶. The molecular structure of levofloxacin is illustrated in Figure 3.40.

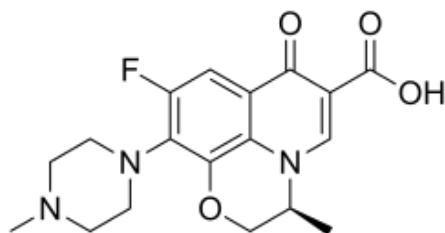


Figure 3. 40: Chemical structure of levofloxacin

3.4.1 Cyclic voltammetric study of levofloxacin

A cyclic voltammetric investigation was conducted using a PGA-modified glassy carbon electrode in phosphate buffer solutions across a pH range of 4.5 to 7.4. The electrochemical measurements were performed within a potential window of 0.0 V to +1.5 V, examining various scan rates and analyte concentrations. Prior to analysis, the solubility of levofloxacin was evaluated in phosphate buffers of different pH levels, with optimal solubility observed at neutral pH (6.8). Consequently, the stock solution of levofloxacin was prepared in phosphate buffer at pH 6.8 for subsequent electrochemical studies.

As a part of electrochemical set up, PGA modified GCE was used as working electrode, Ag/AgCl was used as reference electrode and platinum electrode was used as counter electrode and the potential window maintained from 0.0 V to +1.5 V.

3.4.1.1 Study of redox behavior of levofloxacin in phosphate buffer at different pH

To observe the redox behavior of levofloxacin, a series of cyclic voltammograms were taken with 20 ppb levofloxacin at 50 mV/s scan rate within the switching potential 0.0 V to +1.5 V vs. Ag/AgCl, which are shown in Figure 3.41. Peak current and pH variation are tabulated in Table 3.30.

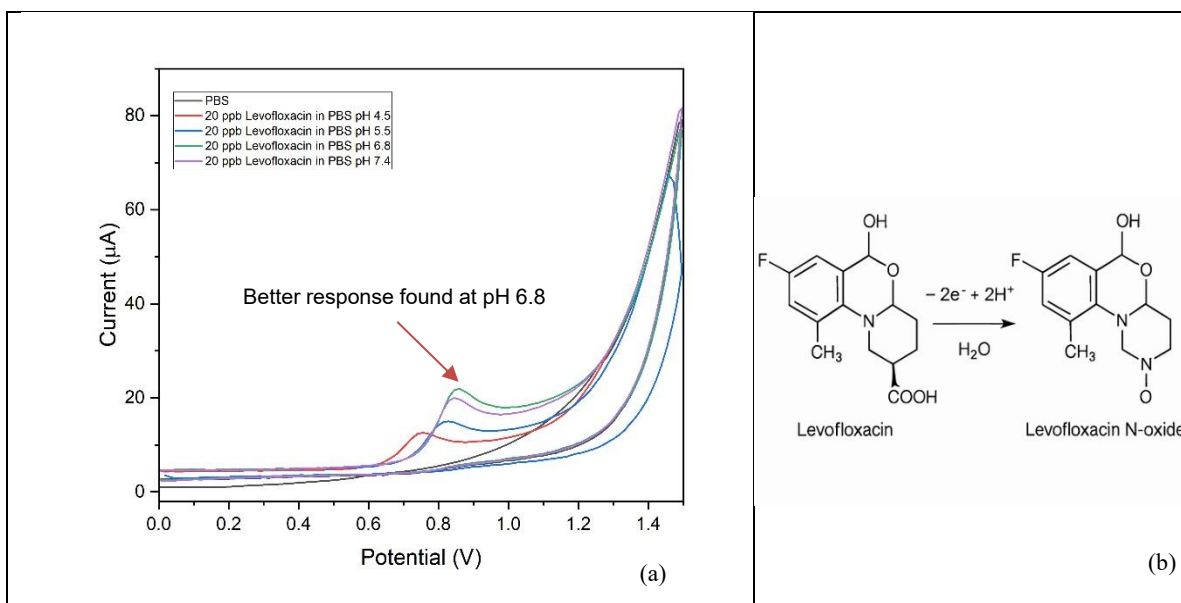


Figure 3. 41: Electrochemical responses and reactions happened during CV run.

- a) Cyclic voltammograms of 20 ppb levofloxacin in phosphate buffer at different pH using PGA modified GCE b) Electrochemical reaction happened in levofloxacin

Cyclic voltammograms were recorded for a 20 ppb Levofloxacin solution within the potential range of 0 V to 1.5 V, employing a poly glutamic acid (PGA)-modified glassy carbon electrode (GCE). A platinum (Pt) electrode served as the counter electrode, while an Ag/AgCl electrode functioned as the reference electrode. Analysis of the overlaid plot indicated an optimal response at pH 6.8.

Figure 3.41 illustrates that; there is a single anodic peak found for each of the voltammogram between +0.7 V to +0.9 V which is oxidation peak happened due to the oxidation and forms N-oxide with levofloxacin. It is an irreversible reaction happened during this electrochemical process. Analysis of the overlaid plot revealed an enhanced electrochemical response (Table 3.30) in phosphate buffer at pH 6.8. From the voltammograms, better response found at pH 6.8 and the peak current found 21.9 μA.

Table 3.30: Variation of peak current with different pH at 50 Scan Rate

pH	Peak Current (μA)
4.5	12.6
5.5	14.8
6.8	21.9
7.4	20.0

3.4.1.2 Study of minimum detection level of levofloxacin using PGA modified GCE

After getting the suitable pH of phosphate buffer, studied the minimum detection level of levofloxacin using PGA modified GCE. For this purpose, CV were taken for 1 ppb, 3 ppb, 5 ppb, 10 ppb and 20 ppb of levofloxacin solutions in PBS at pH 6.8. The voltammograms are overlaid and shown in Figure 3.42.

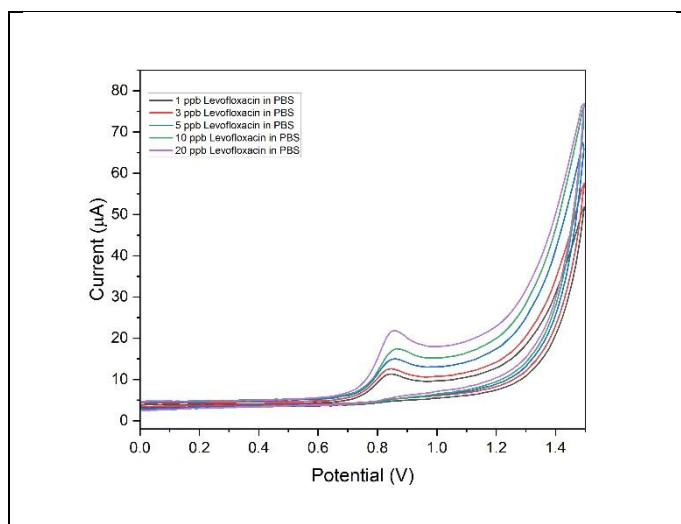


Figure 3. 42: Cyclic voltammograms of different concentrations of levofloxacin in PBS with pH 6.8 using PGA modified GCE at 50 mV/s scan rate

Based on the data obtained from voltammograms, response (peak current) is observed even from 1 ppb levofloxacin solution. So, it is assumed it would be capable to detect a trace level of levofloxacin if present in chemical or biological sample like blood sample.

3.4.1.3 Accuracy of cyclic voltammetric method for levofloxacin determination

Cyclic voltammograms (Figure-3.43) were taken for 3 ppb, 5 ppb, 10 ppb, 20 ppb and 30 ppb solutions of levofloxacin in PBS with pH 6.8 using PGA modified glassy carbon electrode.

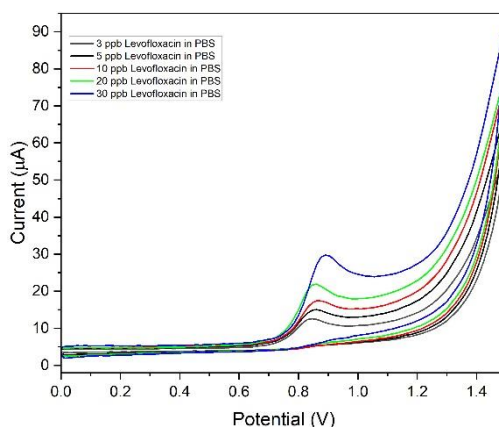


Figure 3. 43: Electrochemical responses (CV) of 3 ppb, 5 ppb, 10 ppb, 20 ppb and 30 ppb solutions of levofloxacin in PBS with pH 6.8 at 50 mV/s scan rate

Table 3.31: Electrochemical responses (peak current) of different concentration of levofloxacin at 50 mV/s scan rate

Concentration in ppb	Peak Current (μA)
3	12.6
5	15.0
10	17.44
20	22.9
30	29.7

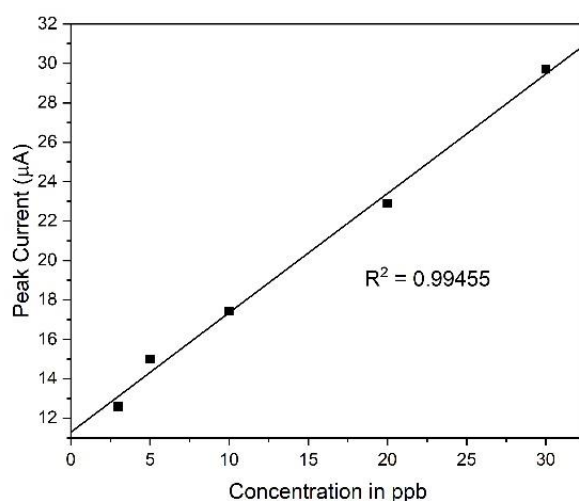


Figure 3. 44: Relationship showing linearity of CV method based on different concentration of levofloxacin

Data obtained from the voltammograms are captured in Table-3.31 and plotted graph in Figure 3.44. From above linearity curve, correlation coefficient found above 0.99455 which shows a satisfactory accuracy and capability of detection. The peak potential has slightly shifted towards the anodic position with the increasing of the concentration of levofloxacin indicating irreversible charge transfer of levofloxacin.

3.4.1.4 Effect of scan rate variation on levofloxacin

Cyclic voltammograms were taken from 20 ppb levofloxacin in phosphate buffer solution pH 6.8 using PGA modified glassy carbon electrode by varying scan rate 25, 50, 75 and 100 mV/s within the potential window 0.0 V to +1.5 V. Cyclic voltammograms are shown in Figure 3.45 and peak current with scan rate variation are tabulated in table 3.32.

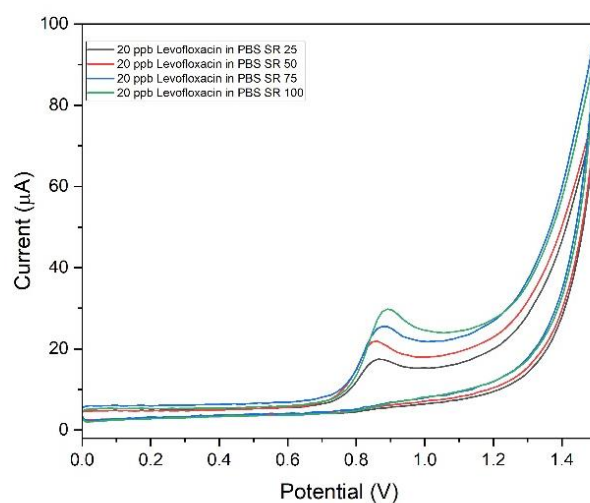


Figure 3. 45: Cyclic voltammograms of 20 ppb levofloxacin in PBS with pH 6.8 at 25, 50, 75 and 100 mV/s scan rate

Table 3.32: Variation of peak current at different scan rate of levofloxacin

Scan Rate	Square Root (SQRT) of Scan Rate	Peak Current (μA)
25	5	17.44
50	7.07107	21.91
75	8.66025	25.53
100	10	29.76

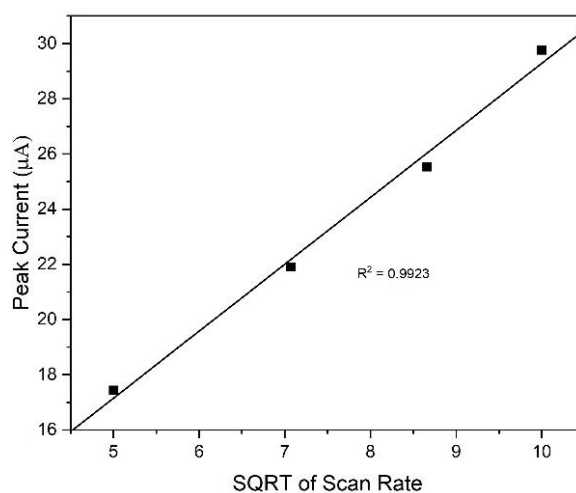


Figure 3. 46: Variation of peak current with SQRT of scan rate

The peak current data obtained from the scan rate variation are tabulated in table-3.32. The calibration plot (Figure-3.46) shows a strong correlation coefficient value which indicates the robustness of the CV test method for levofloxacin determination.

So, based on the above all studies data it can be inferred, PGA modified glassy carbon electrode can determine the levofloxacin reproducibly using CV in phosphate buffer solution pH 6.8.

3.4.2 Linear sweep voltammetric study of levofloxacin

A Linear Sweep voltammetric analysis was conducted utilizing a PGA-modified Glassy Carbon Electrode (GCE) in a Phosphate Buffer Solution at pH 6.8, within a potential range of -0.5 V to +1.6 V. The study was performed at different scan rates and different concentrations. A stock solution was prepared using Phosphate Buffer Solution, and subsequent experiments were carried out in PBS at pH 6.8 within the same potential window while altering the concentration.

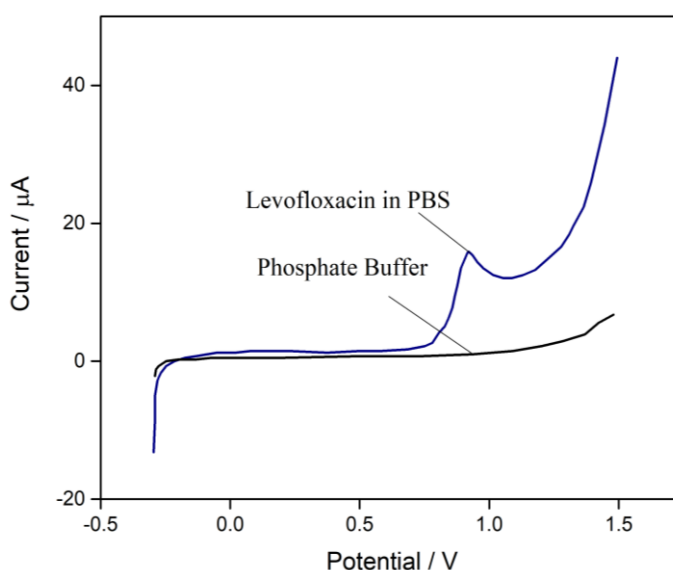


Figure 3. 47: Electrochemical responses (LSV) of 20 ppb solutions of levofloxacin in PBS with pH 6.8 at 50 mV/s scan rate

From the voltammograms (Figure-3.47) it has been observed that, PGA modified GCE is showing prominent response in LSV technique also.

3.4.3 Differential pulse voltammetric study of Levofloxacin

Differential pulse voltammetric study of levofloxacin was conducted using a PGA-modified glassy carbon electrode in phosphate buffer solution pH 6.8. The electrochemical analysis

was performed within a potential window of +0.2 V to +1.3 V, changing both the scan rates and analyte concentrations. The stock solution of levofloxacin was prepared in PBS with pH 6.8, and all experiments were carried out under identical pH conditions of 6.8.

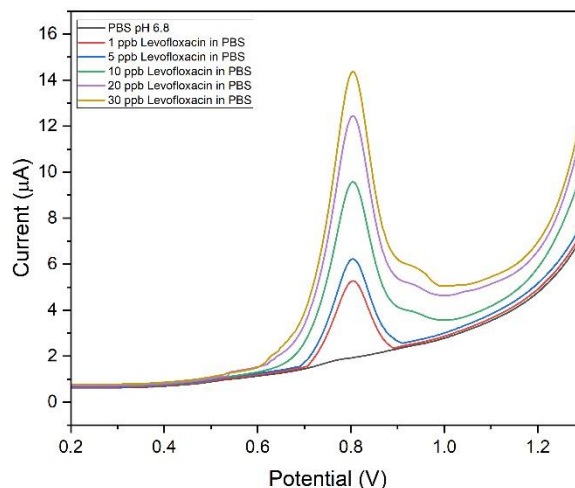


Figure 3. 48: Differential pulse voltammograms of 1 ppb, 5 ppb, 10 ppb, 20 ppb and 30 ppb solutions of levofloxacin in PBS with pH 6.8 at 50 mV/s scan rate

From the voltammograms of Figure 3. 48 it has been observed that, different concentrations of levofloxacin solutions show prominent responses with PGA modified GCE at 50 mV/s scan rate. This indicates a hope to detect a trace level of levofloxacin in real sample (Blood sample) which will help later to determine antibiotic resistance as well.

3.4.4 Recovery study of levofloxacin in blood samples

Blood samples were collected from volunteers, along with relevant medical history and physical conditions data essential to the study.

The study was designed in a sequential step like, 10 mL phosphate buffer pH 6.8 was taken in a cell. Blank run has been captured with CV and DPV technique using PGA modified GCE at 50 mV/s scan rate. After that, added 100 μ L blood serum within phosphate buffer solution and took voltammograms at CV and DPV using PGA modified GCE. Then, added different concentrations of levofloxacin solutions within above mentioned phosphate buffer and blood serum and, took voltammograms at CV and DPV using PGA modified GCE. Finally, solution having 20 ppb levofloxacin kept for more than 24 hours in room temperature around 35°C to 37°C and took voltammograms at CV and DPV using PGA modified GCE at a regular interval up to 24 hours.

3.4.4.1 Study of levofloxacin with blood sample collected from human volunteer-1 by CV technique using PGA modified GCE

Blood sample was collected from human volunteers. Then, centrifuged it to separate blood serum and used blood serum in study. In this study, the volunteers blood group was A (+) ve. His body weight was 78 kg and age was 26 years. The volunteer was the resident of Mirpur, Dhaka.

Cyclic voltammetric study of levofloxacin and blood serum was conducted using PGA-modified glassy carbon electrode in phosphate buffer solution pH 6.8. The electrochemical analysis was performed within a potential window of 0.0 V to +1.5 V at 50 mV/s scan rate. Blank CV run was recorded from 10 mL of phosphate buffer pH 6.8 solution. Then, ran CV scan by adding 100 μ L blood serum. Afterwards, added different concentrations of levofloxacin within it and acquired CV. Also, acquired CV measurements up to 24 hours at a regular interval.

The voltammograms reveal no prominent peak current in blood serum within the potential range, no interference is observed at the potential corresponding to levofloxacin + 0.844 V. The current responses obtained from different concentrations of levofloxacin shows similar accuracy of method observed during the study in phosphate buffer pH 6.8 in absence of blood serum which confirms there is no interference from blood serum. Figure 3. 49 (d) shows that, the peak current responses remain almost constant across different time intervals.

Table 3.33: Peak current response from cyclic voltammograms with time

Volunteer	Potential (V)	Current (μ A)					Difference in Current from T ₀ (μ A)	Remarks
		T ₀	T ₃	T ₅	T ₁₀	T ₂₄		
Volunteer- 1	+0.844	21.42	21.65	21.20	21.34	21.31	-0.11	Peak current remains almost consistent

Based on the above data (Table 3.33) it has been observed that, there was no significant change or degradation in peak current over a period of time. Also understood the stability of the modified glassy carbon electrode that, after modification at least 24 hours it can be used repeatedly.

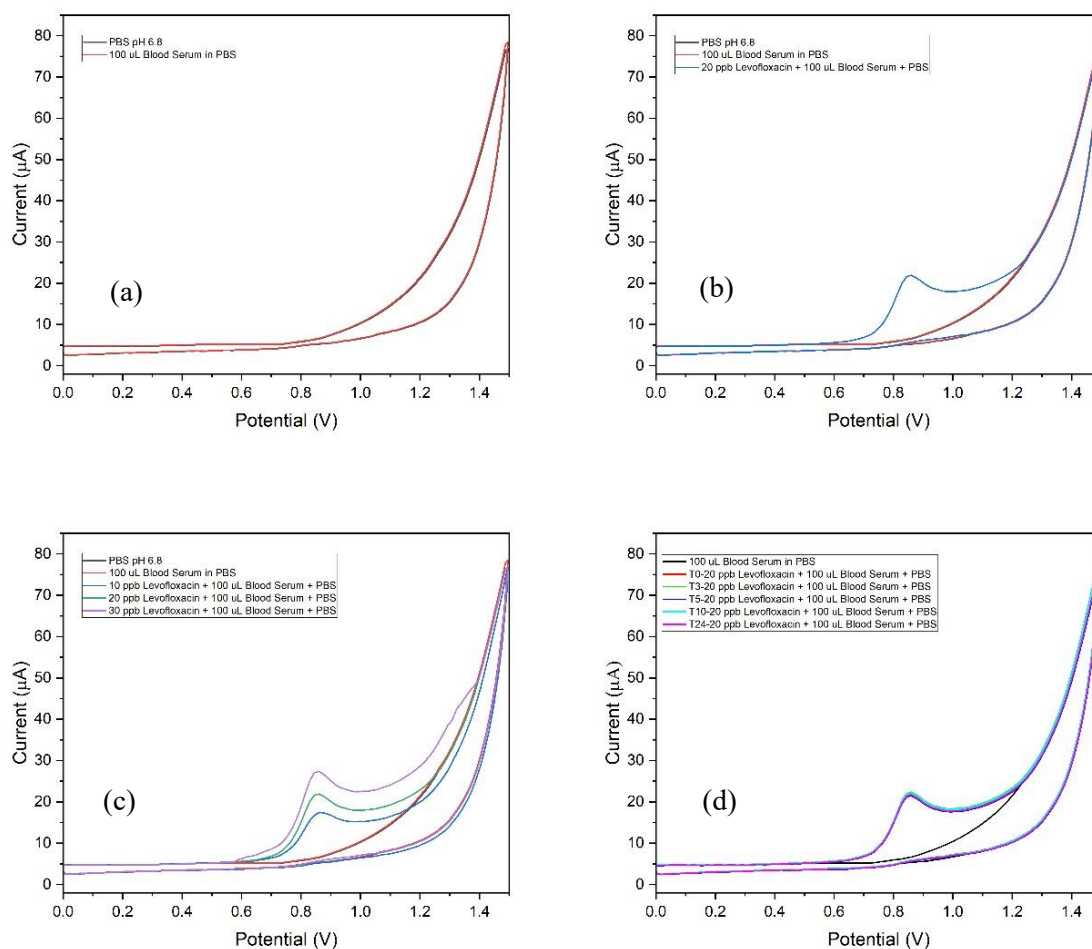


Figure 3. 49: Electrochemical responses recorded from blank, blood serum and different concentration of levofloxacin.

a) Cyclic voltammograms of PBS with pH 6.8 as blank and 100 μL blood serum in PBS with pH 6.8 b) Cyclic voltammograms of 20 ppb levofloxacin in PBS with pH 6.8 with 100 μL blood serum c) Cyclic voltammograms of 10, 20 and 30 ppb levofloxacin in PBS with pH 6.8 with 100 μL blood serum d) Cyclic voltammograms of 20 ppb levofloxacin in PBS with pH 6.8 with 100 μL blood serum at different time interval i.e. initial, after 3, 5, 10 and 24 hours.

3.4.4.2 Study of levofloxacin with blood sample collected from human volunteer-1 by DPV technique using PGA modified GCE

Differential pulse voltammetric study of levofloxacin and blood serum was conducted using PGA-modified glassy carbon electrode in phosphate buffer solution pH 6.8 within the potential window of +0.2 V to +1.3 V at 50 mV/s scan rate. Measurement of blank was acquired from 10 mL of phosphate buffer solution pH 6.8. Then, ran DPV scan by adding

100 μ L blood serum. After that, added different concentrations of levofloxacin within it and acquired DPV. Also, acquired DPV measurements up to 24 hours at a regular interval.

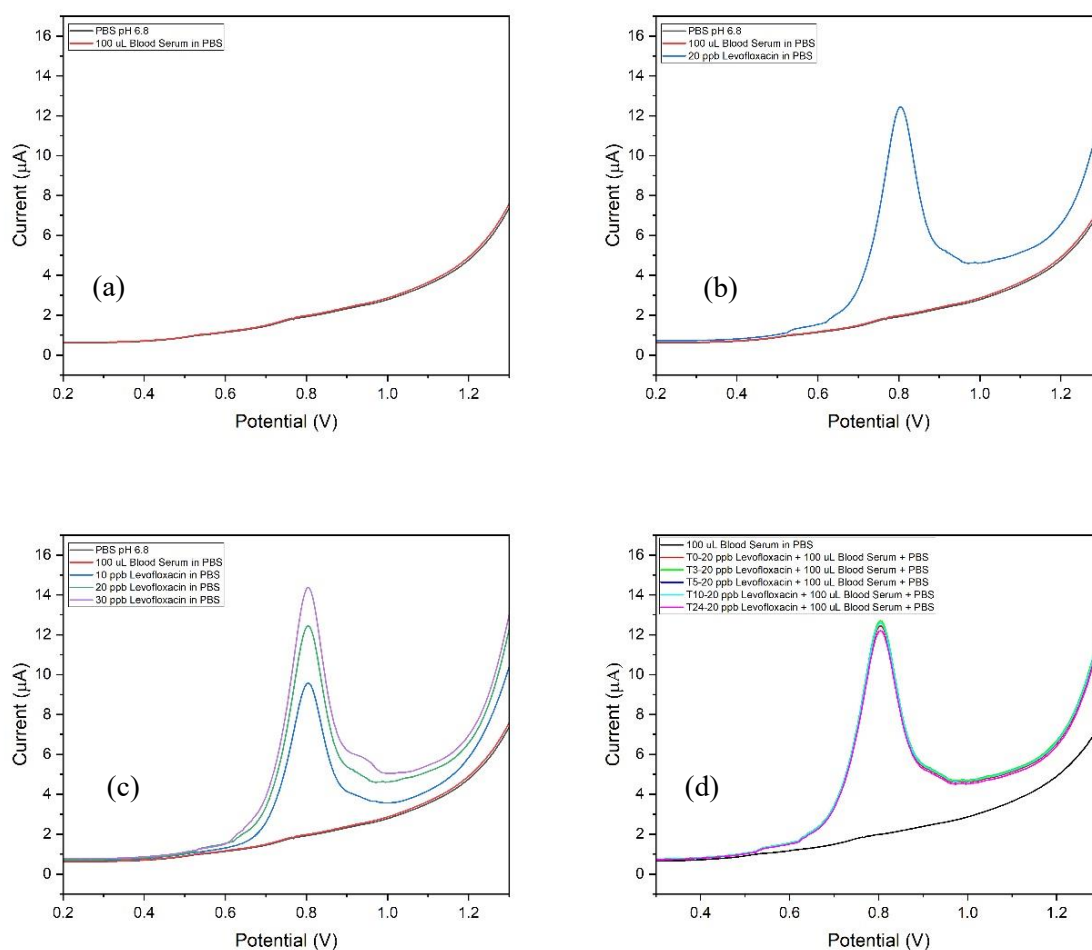


Figure 3. 50: Electrochemical responses recorded from blank, blood serum and different concentration of levofloxacin.

a) Voltammograms of PBS with pH 6.8 as blank and 100 μ L blood serum in PBS with pH 6.8 b) Voltammograms of 20 ppb levofloxacin in PBS with pH 6.8 with 100 μ L blood serum c) Voltammograms of 10, 20 and 30 ppb levofloxacin in PBS with pH 6.8 with 100 μ L blood serum d) Voltammograms of 20 ppb levofloxacin in PBS with pH 6.8 with 100 μ L blood serum at different time interval i.e. initial, after 3, 5, 10 hours and up to 24 hours.

The voltammograms reveal distinct current signal from blood serum at various potentials and no interference is observed at the same potential corresponding to levofloxacin + 0.804 V. The current responses obtained from different concentrations of levofloxacin shows similar accuracy of method observed during the study in phosphate buffer pH 6.8 in absence of blood serum which confirms there is no interference from blood serum.

From Figure 3. 50 (d) observed that, throughout different time intervals, the peak current response remains mostly unchanged.

Table 3.34: Peak potential and current data up to 24 hours

Volunteer	Potential	Current (μA)					Difference in Current from T_0 (μA)	Remarks
	(V)	T_0	T_3	T_5	T_{10}	T_{24}		
Volunteer- 1	+0.804	12.46	12.32	12.55	12.45	12.35	-0.11	Peak current remains almost consistent

Based on the above data (Table 3.34) it has been observed that, there was no significant change in peak current over a period of time.

3.4.4.3 Study of levofloxacin with blood sample collected from human volunteer-2 by CV technique using PGA modified GCE

Blood sample was collected from the volunteer who is a resident of Hajaribag, Dhaka. His blood group is A (+) ve and body weight was 70 kg and age was 20 years old.

The study of levofloxacin and blood serum was conducted using PGA-modified glassy carbon electrode in phosphate buffer solution pH 6.8. The analysis was conducted within a potential window of 0.0 V to +1.5 V at 50 mV/s scan rate. Blank CV run was recorded from 10 mL of phosphate buffer pH 6.8 solution. Then, ran CV scan by adding 100 μL blood serum. Afterwards, added different concentrations of levofloxacin within it and acquired CV. Also, acquired CV measurements up to 24 hours at a regular interval.

From the voltammograms it has been observed that, there are some very low signals in blood serum at various potentials, but no interference is observed at the potential corresponding to levofloxacin + 0.844 V. The current responses obtained from different concentrations of levofloxacin shows similar accuracy of method observed during the study in phosphate buffer pH 6.8 in absence of blood serum which confirms there is no interference from blood serum.

The Figure 3.51 (d) illustrates that, across different time intervals, the peak current response remains nearly unchanged.

From the data (Table 3.35) it has been observed that, there was no significant change in peak current over a period of time.

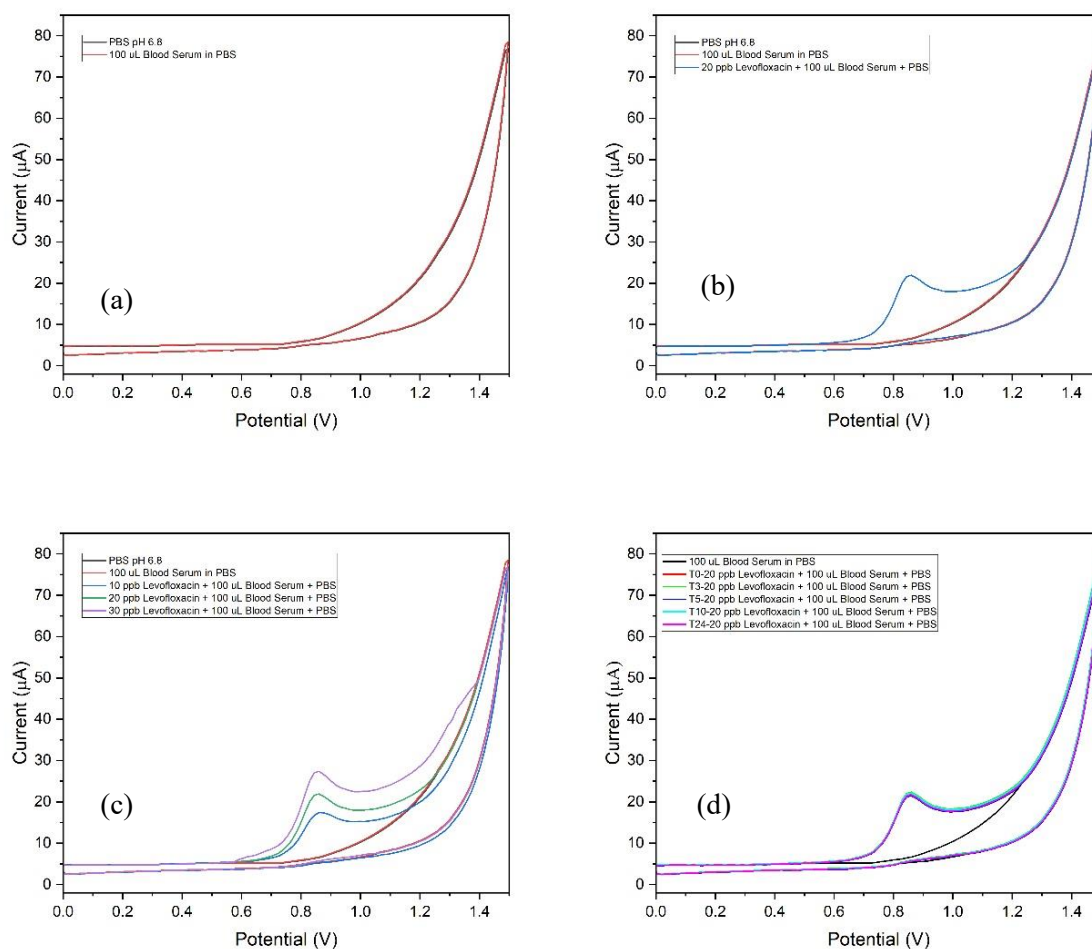


Figure 3. 51: Electrochemical responses recorded from blank, blood serum and different concentration of levofloxacin.

a) Cyclic voltammograms of PBS with pH 6.8 as blank and 100 μL blood serum in PBS with pH 6.8 b) Cyclic voltammograms of 20 ppb levofloxacin in PBS with pH 6.8 with 100 μL blood serum c) Cyclic voltammograms of 10, 20 and 30 ppb levofloxacin PBS with pH 6.8 with 100 μL blood serum d) Cyclic voltammograms of 20 ppb levofloxacin in PBS with pH 6.8 with 100 μL blood serum at different time interval i.e. initial, after 3, 5, 10 and 24 hours.

Table 3.35: Peak current response from cyclic voltammograms with time

Volunteer	Potential (V)	Current (μA)					Difference in Current from T_0 (μA)	Remarks
		T_0	T_3	T_5	T_{10}	T_{24}		
Volunteer-2	+0.844	21.69	21.85	21.55	21.77	21.66	-0.03	Response remains almost consistent

3.4.4.4 Study of levofloxacin with blood sample collected from human volunteer-2 by DPV technique using PGA modified GCE

This electrochemical study of levofloxacin and blood serum was conducted using PGA-modified glassy carbon electrode in phosphate buffer solution pH 6.8 within the potential window of +0.2 V to +1.3 V at 50 mV/s scan rate. Measurement of blank was acquired from 10 mL of phosphate buffer solution pH 6.8. Then, ran DPV scan by adding 100 μ L blood serum. After that, added different concentrations of levofloxacin within it and acquired DPV. Also, acquired DPV measurements up to 24 hours at a regular interval.

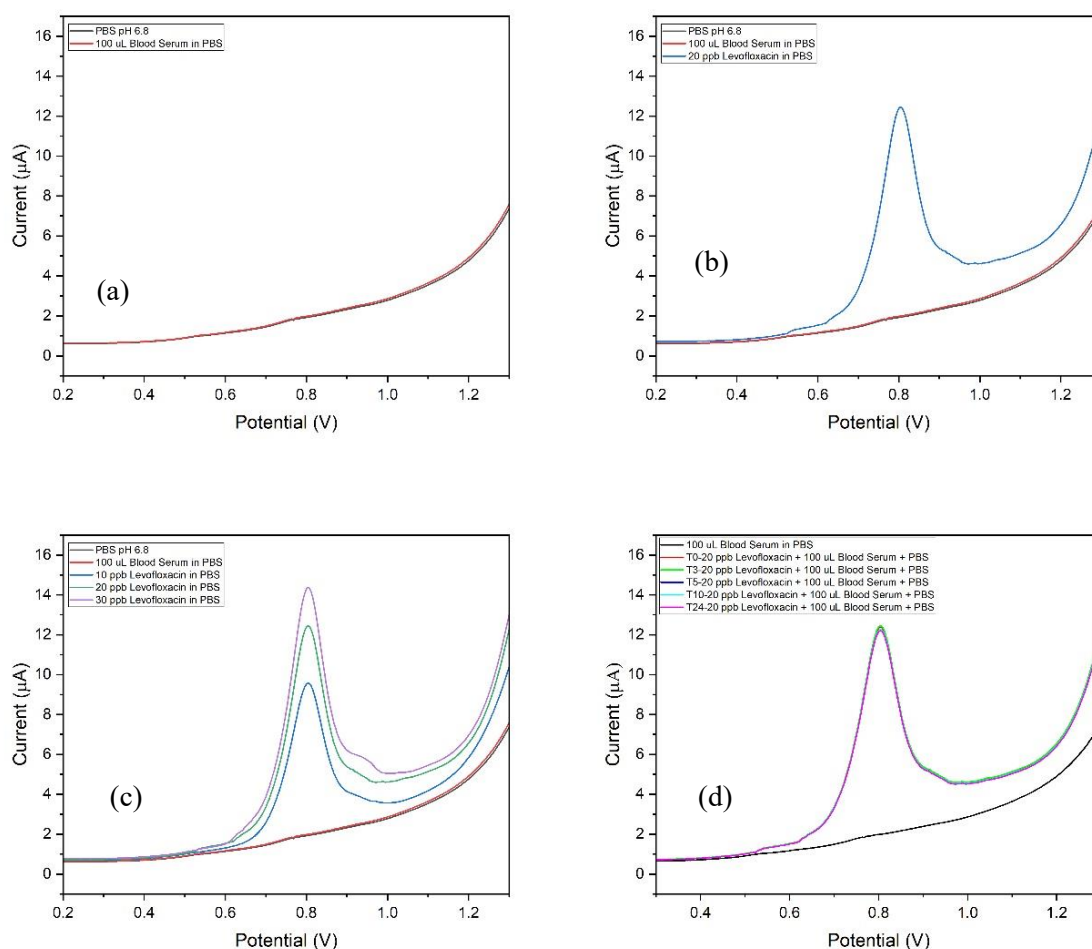


Figure 3. 52: Electrochemical responses recorded from blank, blood serum and different concentration of levofloxacin.

a) Voltammograms of PBS with pH 6.8 as blank and 100 μ L blood serum in PBS with pH 6.8 b) Voltammograms of 20 ppb levofloxacin in PBS with pH 6.8 with 100 μ L blood serum c) Voltammograms of 10, 20 and 30 ppb levofloxacin in PBS with pH 6.8 with 100 μ L blood serum d) Voltammograms of 20 ppb levofloxacin in PBS with pH 6.8 with 100 μ L blood serum at different time interval i.e. initial, after 3, 5, 10 hours and up to 24 hours.

The voltammograms reveal no current signal from blood serum and no interference is observed at the same potential corresponding to levofloxacin + 0.804 V. The current responses obtained from different concentrations of levofloxacin shows similar accuracy of method observed during the study in phosphate buffer pH 6.8 in absence of blood serum which confirms there is no interference from blood serum.

Upon review Figure 3. 52 (d) it has been found that, in different time intervals, the peak current response remains mostly unchanged (Table 3.36).

Table 3.36: Peak current response from differential pulse voltammograms with time

Volunteer	Potential (V)	Current (μ A)					Difference in Current from T ₀ (μ A)	Remarks
		T ₀	T ₃	T ₅	T ₁₀	T ₂₄		
Volunteer-2	+0.804	12.55	12.46	12.35	12.48	12.42	-0.13	Peak current remains almost consistent

No significant change or degradation of levofloxacin happened over a period of time.

3.4.4.5 Study of levofloxacin with blood sample collected from human volunteer-3 by CV technique using PGA modified GCE

As a part of study, collected blood samples from human volunteers. Then, centrifuged it to separate blood serum and used blood serum in study. In this study, the volunteers blood group is A (+) ve. His body weight was 64 kg and age was 22 years. The volunteer was the resident of Shahidullah Hall, Dhaka University.

Cyclic voltammetric study of levofloxacin and blood serum was conducted using PGA-modified glassy carbon electrode in phosphate buffer solution pH 6.8. The electrochemical analysis was performed within a potential window of 0.0 V to +1.5 V at 50 mV/s scan rate. Blank CV run was recorded from 10 mL of phosphate buffer pH 6.8 solution. Then, ran CV scan by adding 100 μ L blood serum. Afterwards, added different concentrations of levofloxacin within it and acquired CV. Also, acquired CV measurements up to 24 hours at a regular interval. The voltammograms reveal distinct current signal peaks in blood serum at various potentials, yet no interference is observed at the potential corresponding to levofloxacin + 0.844 V. The current responses obtained from different concentrations of levofloxacin shows similar accuracy of method observed during the study in phosphate buffer pH 6.8 in absence of blood serum which confirms there is no interference from blood serum.

Figure 3. 53 (d) illustrates that in different time intervals; the peak current response remains almost constant.

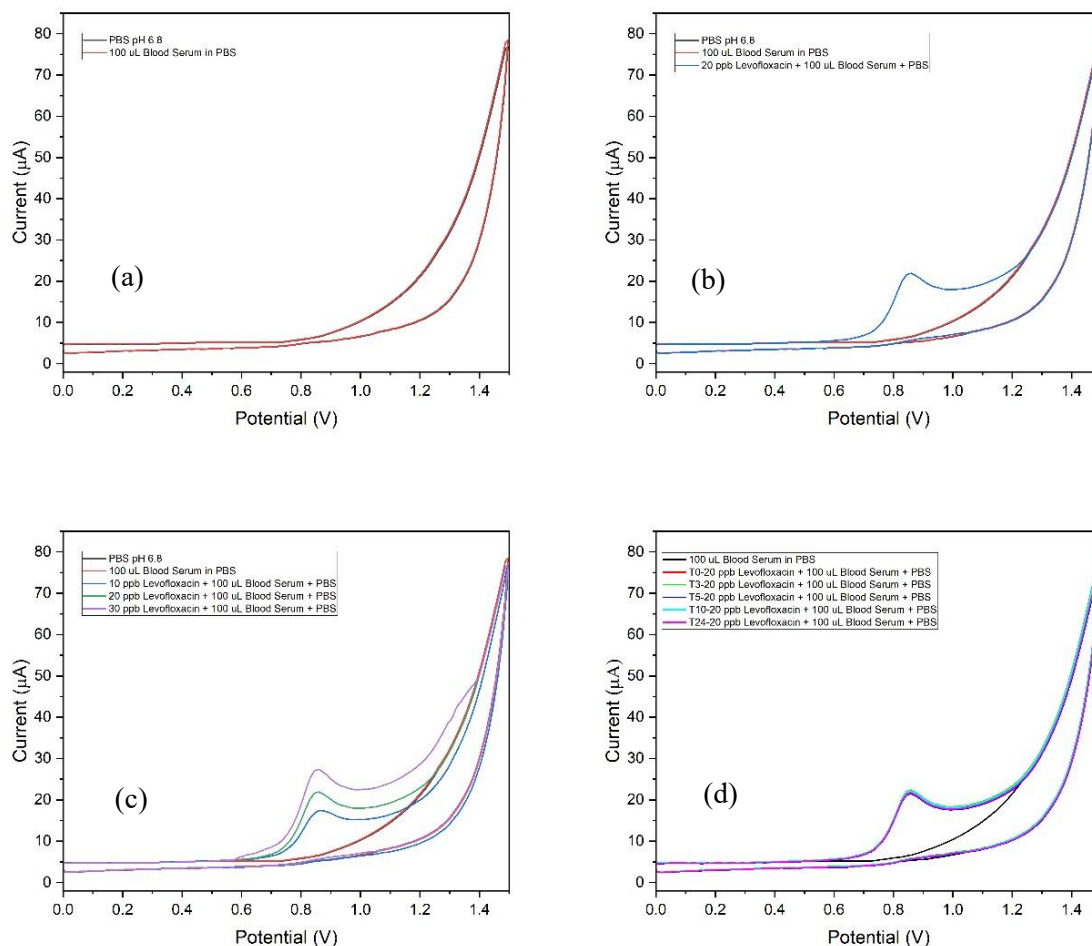


Figure 3. 53: Electrochemical responses recorded from blank, blood serum and different concentration of levofloxacin.

a) Cyclic voltammograms of PBS with pH 6.8 as blank and 100 μ L blood serum in PBS with pH 6.8 b) Cyclic voltammograms of 20 ppb levofloxacin in PBS with pH 6.8 with 100 μ L blood serum c) Cyclic voltammograms of 10, 20 and 30 ppb levofloxacin in PBS with pH 6.8 with 100 μ L blood serum d) Cyclic voltammograms of 20 ppb levofloxacin in PBS with pH 6.8 with 100 μ L blood serum at different time interval i.e. initial, after 3, 5, 10 hours and up to 24 hours.

Table 3.37: Peak current response from cyclic voltammograms with time

Volunteer	Potential (V)	Current (μA)					Difference in Current from T_0 (μA)	Remarks
		T_0	T_3	T_5	T_{10}	T_{24}		
Volunteer-3	+0.844	21.46	21.85	21.20	21.34	21.45	-0.01	No significant change

Peak current response (Table 3.37) remains almost consistent in levofloxacin concentration even after a long time which indicates, levofloxacin couldn't bind with any bacteria.

3.4.4.6 Study of levofloxacin with blood sample collected from human volunteer-3 by DPV technique using PGA modified GCE

Differential pulse voltammetric study of levofloxacin and blood serum was conducted using PGA-modified glassy carbon electrode in phosphate buffer solution pH 6.8 within the potential window of +0.2 V to +1.3 V at 50 mV/s scan rate. Measurement of blank was acquired from 10 mL of phosphate buffer solution pH 6.8. Then, ran DPV scan by adding 100 μL blood serum. After that, added different concentrations of levofloxacin within it and acquired DPV. Also, acquired DPV measurements up to 24 hours at a regular interval.

The voltammograms reveal distinct current signal from blood serum at various potentials and interference is observed at the same potential corresponding to levofloxacin + 0.804 V. The current responses obtained from different concentrations of levofloxacin shows similar accuracy of method observed during the study in phosphate buffer pH 6.8 in absence of blood serum which confirms there is no interference from blood serum. From Figure 3. 54 (d) observed that, across different time intervals, the peak current response remains almost consistent (Table 3.38).

Table 3.38: Peak current response from differential pulse voltammograms with time

Volunteer	Potential (V)	Current (μA)					Difference in Current from T_0 (μA)	Remarks
		T_0	T_3	T_5	T_{10}	T_{24}		
Volunteer-3	+0.804	12.65	12.48	12.34	12.55	12.41	-0.24	No significant change

No significant change or degradation of levofloxacin happened over a period of time.

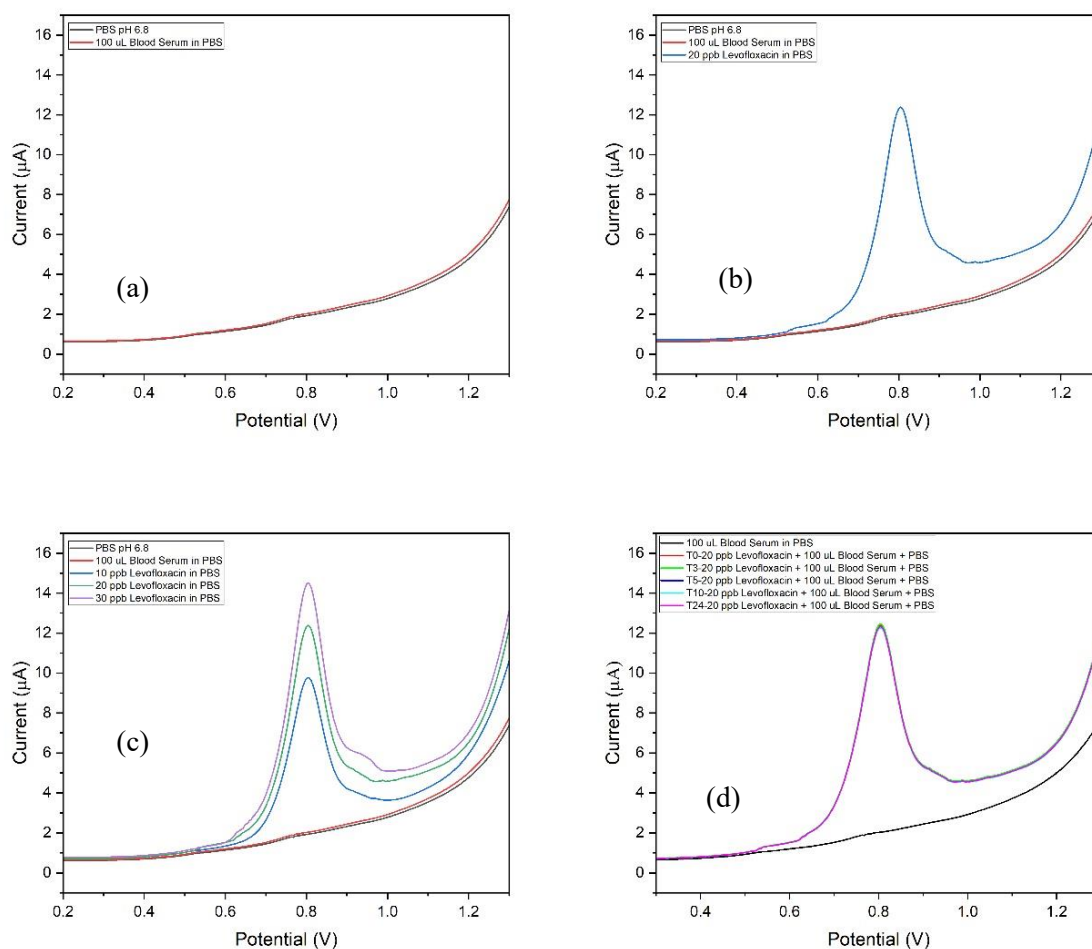


Figure 3. 54: Electrochemical responses recorded from blank, blood serum and different concentration of levofloxacin.

a) Voltammograms of PBS with pH 6.8 as blank and 100 μL blood serum in PBS with pH 6.8 b) Voltammograms of 20 ppb levofloxacin in PBS with pH 6.8 with 100 μL blood serum c) Voltammograms of 10, 20 and 30 ppb levofloxacin in PBS with pH 6.8 with 100 μL blood serum d) Voltammograms of 20 ppb levofloxacin in PBS with pH 6.8 with 100 μL blood serum at different time interval i.e. initial, after 3, 5, 10 hours and up to 24 hours.

3.4.4.7 Summary and conclusion of data obtained from all real (blood) samples:

The potential values and peak currents derived from the voltammograms of both cyclic voltammetry and differential pulse voltammetry are presented in the below Tables 3.39 and 3.40.

Table 3.39: All data obtained from cyclic voltammetric study

Volunteer	Potential	Current (μA)					Difference in Current from T_0 (μA)	Remarks
	(V)	T_0	T_3	T_5	T_{10}	T_{24}		
Volunteer-1	+0.844	21.42	21.65	21.20	21.34	21.31	-0.11	No significant change
Volunteer-2	+0.844	21.69	21.85	21.55	21.77	21.66	-0.03	
Volunteer-3	+0.844	21.46	21.85	21.20	21.34	21.45	-0.01	

Table 3.40: All Data obtained from differential pulse voltammetric study

Volunteer	Potential	Current (μA)					Difference in Current from T_0 (μA)	Remarks
	(V)	T_0	T_3	T_5	T_{10}	T_{24}		
Volunteer-1	+0.804	12.46	12.32	12.55	12.45	12.35	-0.11	No significant change
Volunteer-2	+0.804	12.55	12.46	12.35	12.48	12.42	-0.13	
Volunteer-3	+0.804	12.65	12.48	12.34	12.55	12.41	-0.24	

From the above data summary, current response remains almost consistent or no change happened in levofloxacin concentration even after a long time which indicates, levofloxacin didn't bind with any bacteria or no degradation happened. It can be happened in ways. First, there was no sensitive bacteria within the blood serum, so no levofloxacin binds with bacteria DNA. Or, second, based on the mechanism of action of levofloxacin, there were resistant microorganism so levofloxacin couldn't bind. The first option then challenged by applying ATCC culture having *E. coli* (Gram negative bacteria) within the blood serum and then added levofloxacin and outcome is explained below.

3.4.5 In vitro study of antibiotic resistance against levofloxacin using *E. coli* ATCC strains

Based on the mechanism of levofloxacin and *E. coli* ATCC 8739, if added ATCC 8739 (*Escherichia coli*, a standard strain used in microbiological testing) to a levofloxacin solution containing blood serum and phosphate buffer (pH 6.8), several key interactions and outcomes can be expected. Considering the bacterial growth and antibiotic effect, levofloxacin is a fluoroquinolone antibiotic that inhibits bacterial DNA gyrase and topoisomerase IV, leading to cell death. On the other hand, ATCC 8739 (*E. coli*) is typically

susceptible to levofloxacin, so its growth will likely be inhibited or killed, depending on levofloxacin concentration if at or above the minimum inhibitory concentration (MIC) for *E. coli*. In addition, exposure time can impact like longer exposure increases bacterial death and serum and buffer effects.

So, considering the above mechanisms, study was designed in such a way so that bacterial growth can be studied by applying below the minimum inhibitory concentration (MIC) of levofloxacin into the solution having ATCC 8739 (*E. coli*), phosphate buffer pH 6.8 and blood serum. In this case, concentration of levofloxacin will not remain fully constant. It will gradually decrease due to:

- (i) Serum protein binding (immediate)
- (ii) Bacterial uptake (over hours)
- (iii) Slow degradation (days)

Another study was designed in such a way so that, bacterial death can also be studied by applying above the minimum inhibitory concentration (MIC) of levofloxacin into the solution having ATCC 8739 (*E. coli*), phosphate buffer pH 6.8 and blood serum. In this case, bactericidal effect will happen like cell death, not just growth inhibition. Levofloxacin may cause almost 100% reduction of susceptible *E. coli* in viable CFUs within a few hours. So, concentration of levofloxacin will degrade a certain amount and then it will remain be constant at least a period of time like several hours until levofloxacin can degrade due to the serum protein binding or slow degradation due to the solution instability.

3.4.5.1 Study of levofloxacin (below MIC) with blood serum and *E. coli* ATCC strains by CV technique using PGA modified GCE

As a part of this study, again collected blood samples from another volunteer. Then, centrifuged it to separate blood serum and used blood serum in study. In this study, the volunteers blood group is A (+) ve. His body weight was 78 kg and age was 26 years. The volunteer was the resident of Mirpur, Dhaka.

Cyclic voltammetric study of levofloxacin with blood serum and *E. coli* ATCC strain was conducted using PGA-modified glassy carbon electrode in phosphate buffer solution pH 6.8. The electrochemical analysis was performed within a potential window of +0 V to +1.5 V at 50 mV/s scan rate. Blank CV run was recorded from 10 mL of phosphate buffer pH 6.8 solution. Then, ran CV scan by adding 300 μ L blood serum. Afterwards, added 10 ppb of

levofloxacin within it and acquired CV. Finally, gradually added 100 μ L, 200 μ L and 300 μ L *E. coli* ATCC strain (100 μ L ATCC is equivalent to 10 to 100 cfu) within it and took CV run. Acquired CV measurements at least 30 minutes later after adding ATCC strains.

Table 3.41: Peak potential and peak current data obtained from different voltammograms

Time	Potential (V)	Peak Current (μ A)
10 ppb Levofloxacin (before adding ATCC)	+0.862	17.45
After adding 100 μ L <i>E. coli</i> ATCC	+0.861	14.89
After adding 200 μ L <i>E. coli</i> ATCC	+0.845	12.60
After adding 300 μ L <i>E. coli</i> ATCC	+0.845	11.35
After 24 hours	+0.845	10.66

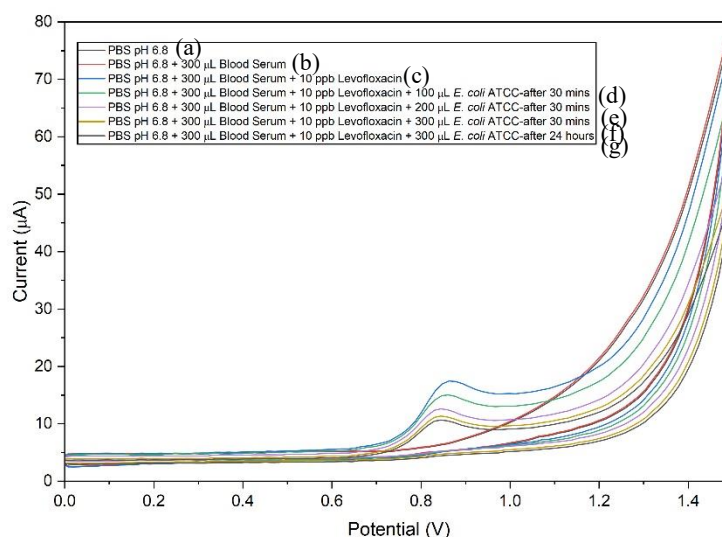


Figure 3. 55: Electrochemical responses recorded from PBS with pH 6.8 as blank, after adding 300 μ L blood serum, 10 ppb of levofloxacin and different concentration of *E. coli* ATCC.

a) Voltammograms of PBS with pH 6.8 as blank b) 300 μ L blood serum in PBS with pH 6.8 c) Voltammograms of 10 ppb levofloxacin in PBS with pH 6.8 with 300 μ L blood serum d) Voltammograms of 10 ppb levofloxacin in PBS with pH 6.8 with 100 μ L blood serum and after adding 100 μ L *E. coli* ATCC e) Voltammograms of 10 ppb levofloxacin in PBS with pH 6.8 with 300 μ L blood serum and after adding 200 μ L *E. coli* ATCC f) Voltammograms of 10 ppb levofloxacin in PBS with pH 6.8 with 300 μ L blood serum and after adding 300 μ L *E. coli* ATCC g) Acquired voltammogram after 24 hours from same retained solution.

From the voltammograms Figure 3. 55 and Table 3. 41, it reveals, as the concentration of levofloxacin was very low compared to the *E. coli* strain concentration so, peak response of levofloxacin decreases after gradual increase of *E. coli* ATCC due to the bacterial uptake and serum protein binding. Also, with respect to the time like after 24 hours it degrades a few more quantity might be due to the solution instability. These consequences can be compared to the antibiotic resistance where bacterial growth will remain continue and slowly the concentration of antibiotic will decay towards nil.

3.4.5.2 Study of levofloxacin (below MIC) with blood serum and *E. coli* ATCC 8739 strains by DPV technique using PGA modified GCE

Differential pulse voltammetric study of levofloxacin and blood serum was conducted using PGA-modified glassy carbon electrode in phosphate buffer solution pH 6.8 within the potential window of +0.2 V to +1.3 V at 50 mV/s scan rate. Measurement of blank was acquired from 10 mL of phosphate buffer solution pH 6.8. Then, ran DPV scan by adding 300 μ L blood serum. Afterwards, added 10 ppb of levofloxacin within it and acquired DPV. Finally, gradually added 100 μ L, 200 μ L and 300 μ L *E. coli* ATCC strains within it and acquired DPV run. Also, acquired DPV measurements at different time point to see the impact.

Table 3.42: Peak potential and peak current data obtained from differential pulse voltammetric study

Time	Potential (V)	Peak Current (μ A)
Initial (before adding <i>E. coli</i> ATCC)	+0.804	8.01
After adding 100 μ L <i>E. coli</i> ATCC	+0.804	5.72
After adding 200 μ L <i>E. coli</i> ATCC	+0.804	5.17
After adding 300 μ L <i>E. coli</i> ATCC	+0.804	4.65
After 24 hours	+0.804	4.27

From the voltammograms Figure 3. 56 reveal, as the concentration of levofloxacin was very low compared to the *E. coli* strains concentration so, peak response (Table 3.42) of levofloxacin decreased after gradual increase of *E. coli* ATCC due to the bacterial uptake and serum protein binding. Also, with respect to the time like after 24 hours it degrades a few more quantity might be due to the solution instability.

These consequences can be compared to the antibiotic resistance where bacterial growth will remain continue and slowly the concentration of antibiotic will decay.

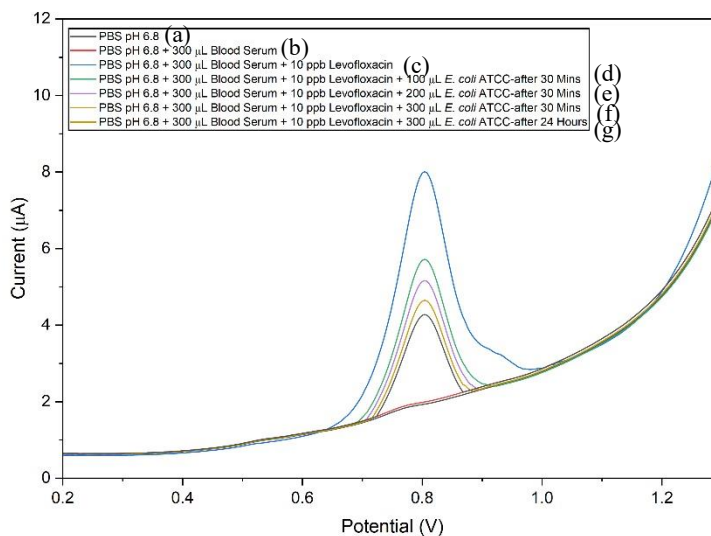


Figure 3. 56: Electrochemical responses recorded from PBS with pH 6.8 as blank, after adding 300 μL blood serum, 10 ppb of levofloxacin and different concentration of *E. coli* ATCC.

a) Voltammograms of PBS with pH 6.8 as blank b) 300 μL blood serum in PBS with pH 6.8 c) Voltammograms of 10 ppb levofloxacin in PBS with pH 6.8 with 300 μL blood serum d) Voltammograms of 10 ppb levofloxacin in PBS with pH 6.8 with 300 μL blood serum and after adding 100 μL *E. coli* ATCC e) Voltammograms of 10 ppb levofloxacin in PBS with pH 6.8 with 300 μL blood serum and after adding 200 μL *E. coli* ATCC f. Voltammograms of 10 ppb levofloxacin in PBS with pH 6.8 with 300 μL blood serum and after adding 300 μL *E. coli* ATCC g. Voltammogram acquired after 24 hours from retained solution.

3.4.5.3 Study of levofloxacin (above MIC) with blood serum and *E. coli* ATCC strains by CV technique using PGA modified GCE

As a part of this study, again collected blood samples from another volunteer. Then, centrifuged it to separate blood serum and used blood serum in study. In this study, the volunteers blood group is A (+) ve. His body weight was 78 kg and age was 26 years. The volunteer was the resident of Mirpur, Dhaka.

Cyclic voltammetric study of levofloxacin with blood serum and *E. coli* ATCC strain was conducted using PGA-modified glassy carbon electrode in phosphate buffer solution pH 6.8. The electrochemical analysis was performed within a potential window of +0 V to +1.5 V at 50 mV/s scan rate. Blank CV run was recorded from 10 mL of phosphate buffer pH 6.8 solution. Then, ran CV scan by adding 300 μL blood serum. Afterwards, added 30 ppb of

levofloxacin within it and acquired CV. Finally, added 10 μL *E. coli* ATCC strains (equivalent to 1 to 10 cfu) within it and took CV run. Acquired CV measurements after 30 minutes, after 2 hours and after 4 hours after adding ATCC strains.

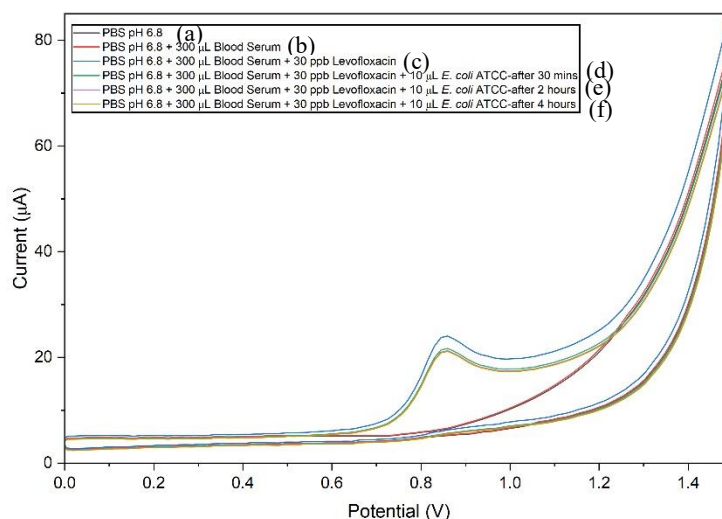


Figure 3. 57: Electrochemical responses recorded from PBS with pH 6.8 as blank, after adding 300 μL blood serum, 10 ppb of levofloxacin and 10 μL *E. coli* ATCC strains

a) Voltammograms of PBS with pH 6.8 as blank b) 300 μL blood serum in PBS with pH 6.8 c) Voltammograms of 30 ppb levofloxacin in PBS with pH 6.8 with 300 μL blood serum d) Voltammograms of 30 ppb levofloxacin in PBS with pH 6.8 with 300 μL blood serum and after 30 minutes, 2 hours and 4 hours of adding 10 μL *E. coli* ATCC strains.

Table 3.43: Peak potential and peak current data obtained from different voltammograms

Time	Potential (V)	Peak Current (μA)
Initial (before adding <i>E. coli</i> ATCC)	+0.859	24.05
After adding 100 μL <i>E. coli</i> ATCC	+0.861	21.42
After adding 200 μL <i>E. coli</i> ATCC	+0.845	21.15
After adding 300 μL <i>E. coli</i> ATCC	+0.845	21.15
After 24 hours	+0.845	21.14

From the voltammograms (Figure 3. 57) reveal, as the concentration of levofloxacin was high compared to the *E. coli* strains concentration so, the microorganisms were killed and a certain amount of levofloxacin was used for that. After that the current (Table 3.43) remained almost consistent up to 4 hours.

3.4.5.4 Study of levofloxacin (above MIC) with blood serum and *E. coli* ATCC 8739 strain by DPV technique using PGA modified GCE

Differential pulse voltammetric study of levofloxacin and blood serum was conducted using PGA-modified glassy carbon electrode in phosphate buffer solution pH 6.8 within the potential window of +0.2 V to +1.3 V at 50 mV/s scan rate. Measurement of blank was acquired from 10 mL of phosphate buffer solution pH 6.8. Then, ran DPV scan by adding 300 μ L blood serum. Afterwards, added 10 ppb of levofloxacin within it and acquired DPV. Finally added 10 μ L *E. coli* ATCC strains within it and took DPV run up to 4 hours.

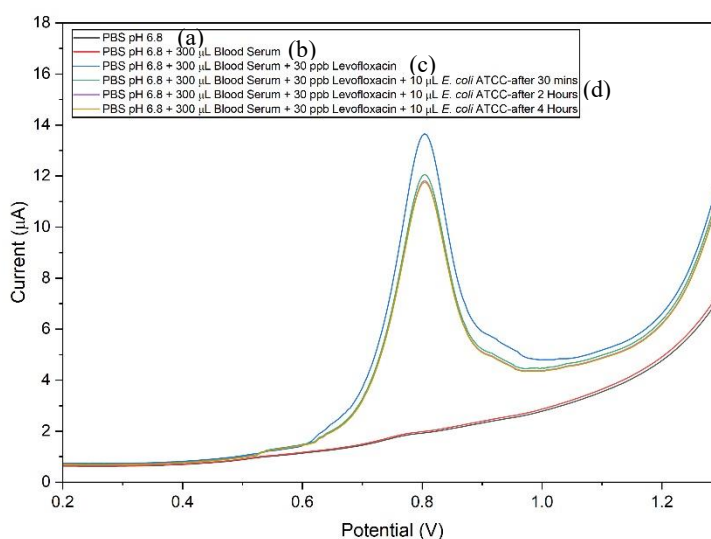


Figure 3. 58: Electrochemical responses recorded from PBS with pH 6.8 as blank, after adding 300 μ L blood serum, 10 ppb of levofloxacin and 10 μ L of *E. coli* ATCC strains.

a) Voltammograms of PBS with pH 6.8 as blank b) 300 μ L blood serum in PBS with pH 6.8 c. Voltammograms of 10 ppb levofloxacin in PBS with pH 6.8 with 300 μ L blood serum d) Voltammograms of 10 ppb levofloxacin in PBS with pH 6.8 with 100 μ L blood serum and after adding 10 μ L *E. coli* ATCC strains after 30 minutes, 2 hours and 4 hours

Table 3.44: Data obtained from differential pulse voltammetric study

Time	Potential (V)	Peak Current (μ A)
Initial (before adding <i>E. coli</i> ATCC)	+0.804	13.65
After adding 100 μ L <i>E. coli</i> ATCC	+0.804	12.06
After adding 200 μ L <i>E. coli</i> ATCC	+0.804	11.82
After adding 300 μ L <i>E. coli</i> ATCC	+0.804	11.76
After 24 Hours	+0.804	11.75

From the voltammograms (Figure 3. 58) reveal, as the concentration of levofloxacin was high compared to the *E. coli* ATCC strains concentration so, the microorganisms were killed and a certain amount of levofloxacin was used for that. After that the concentration (Table 3.44) remained almost consistent up to 4 hours.

CHAPTER IV

CONCLUSION

CHAPTER IV: CONCLUSION

4.1 Conclusion

- We have successfully developed the method for the quantitative determination of antibiotics ceftibuten, cefuroxime and levofloxacin using PGA modified glassy carbon electrode in phosphate buffer at biological pH as well as the sample spiking with blood serum.
- All of the antibiotics ceftibuten, cefuroxime and levofloxacin give an oxidation peak in cyclic voltammograms and differential pulse voltammograms. The peak is more prominent in DPV for all of above three molecules. From the peak current qualitative and quantitative determination of the mentioned antibiotics were possible.
- From the simulated In-vitro study, it has been observed that, the developed electrochemical method can determine the gradual degradation of β -lactam ring of cephalosporin antibiotics after adding β -lactamase which can indicate the resistant properties of the microorganisms.
- Similarly, the method is capable to detect and quantify the quinolone antibiotic, when bacterial culture strain (*E. coli* ATCC 8739) is added that can indicate same sensitivity or resistant properties.
- By comparative study with non-electrochemical techniques, it can be concluded that, by using our developed PGA modified GCE electrochemical system is less time-consuming technique relative to other conventional techniques to study the antibiotic resistance capability of the respective drugs. It would be capable to determine antibiotic resistance preferably within 2 to 3 hours, whereas in other techniques need 18 to 20 hours and a huge cost for sophisticated instrument-based techniques which is not feasible for mass people.
- From the overall research, we can conclude that, the PGA modified GCE system would be promising to explore the proposed rapid testing technique for the antimicrobial susceptibility testing towards antibiotic resistance to get faster result.
- In this research, studies were conducted simulating the human biological systems as In-vitro study. Also, in-parallel study of electrochemical vs microbiological/ others were not performed. So future studies can be conducted in-parallel with microbiological/ others techniques to get comprehensive data which will help to take decision about the suitability and superiority of electrochemical techniques. As well as studies then can be conducted in biological system as clinical study.

- Studies were conducted for only two antibiotics, ceftibuten and cefuroxime from cephalosporin group and one molecule levofloxacin from quinolone group. Studies can extend to other drug molecules of the same groups.
- Suitable electrochemical method can be developed for other groups of antimicrobial molecules also.

CHAPTER V

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CHAPTER V: REFERENCES

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Ethical Clearance

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September 25, 2024

Ethical Review Committee

Professor Dr. Md. Qamrul Ehsan
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Sub: Ethical Clearance

Dear Dr. Md. Qamrul Ehsan,

With reference to your application on the above subject, this is to inform you that your research proposal entitled "Development of Rapid Testing Technique to Diagnose Antibiotic Resistance" has been reviewed and approved by the Ethical Review Committee of the Faculty of Biological Sciences, University of Dhaka.

I wish for the success of your research project.

Akhter
25/9/24

Professor Dr. Md. Akhter Hossain Khan
Dean (Acting), Faculty of Biological Sciences
University of Dhaka