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Application of electrochemical based sensors in support of Toxicokinetic and Pharmacokinetics

By

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This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.

Signed: **Date:**

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Abbreviations

ACN	Acetonitrile
ABs	Antibiotics
AGs	Aminoglycosides
AME	Aminoglycoside modifying enzymes
AMR	Antimicrobial resistance
β-lactam	Beta-lactam
BEH	Bridged Ethylene Hybrid
CSH	Charged Surface Hybrid
CE	Collision Energy
CID	Collision-induced dissociation
cps	Counts per second
CE	Counter Electrode
CL	Chemiluminescence
CV	Cyclic Voltammetry
E-AB	Electrochemical aptamer-based
ECL	Electrochemiluminescence
ESI	Electrospray ionisation
FA	Formic acid
HFBA	Heptafluorobutyric
HPLC	High-performance liquid chromatographic
HOMO	Highest Occupied Molecular Orbital
IPR	Ion-pair reagent
IS	Internal standard
LC	Liquid chromatography
LC-MS/MS	liquid chromatography tandem mass spectrometry
LLQ	Lowest limit of quantification
LOD	Limit of detection
LOMO	Lowest Unoccupied Molecular Orbital
m/z	mass-to-charge
MeOH	Methanol
MOA	Mechanism of action
MRM	Multiple reaction monitoring
ms	Milliseconds
MS/MS	Tandem mass spectrometry
MW	Molecular Weight
P&A	Precision and accuracy
PAE	Post-antibiotic effect
PD	Pharmacodynamics
PK	Pharmacokinetics
PPT	Protein precipitation
QC	Quality control
RE	Reference Electrode

RP	Reverse phase
RT	Retention time
S/N	Signal to Noise
SPE	Solid Phase Extraction
SPE	Screen Printed Electrodes
TFA	Trifluoroacetic
TK	Toxicokinetic
WE	Working Electrode

Abstract

Presently, the capability of electrochemical aptamer-based sensors (E-AB) are being explored, which incorporate aptamers for sensing small molecules and proteins, transducing the signal electrochemically. Currently, much work around E-ABs is being conducted by a group at the University of California, Santa Barbara for recognising aminoglycoside antibiotics, inclusive of tobramycin. To validate the accuracy of the biosensor measurements, a proof of concept is required to compare E-AB results with traditional samples. For this there is a need for a simple, reliable and sensitive method for quantification of tobramycin. Two techniques were explored, resulting in different endpoints of a quick qualitative measurement, compared to a more quantitative result.

Firstly, a liquid chromatography tandem mass spectrometry (LC-MS/MS) assay has been successfully developed for the determination of tobramycin in rat plasma, over a linear dynamic range of 50 - 10,000 ng/mL, using protein precipitation (PPT) and multiple reaction monitoring (MRM). Secondly, as an alternative method of detection, electrochemiluminescence (ECL) was investigated to detect tobramycin using screen printed electrodes (SPE) modified with a nafion-ruthenium film, down to 2.34 µM.

Overall, ECL showed promise as a detection method and as a technique for screening, providing a qualitative assessment for the presence of tobramycin. Further work is required, however in the future LC-MS/MS could be utilised to provide a quantitative result for tobramycin in rat plasma, after initial ECL measurements. These techniques could be used in the future to support output of biosensor measurements.

Overview

The availability of a technology to provide real-time measurements of small molecules in living organisms would have the potential to improve our knowledge on pharmacokinetics (PK) and toxicokinetic (TK), with the long-term potential of therapeutic drug monitoring [1]. E-ABs are proposed for this and incorporate aptamers, which are chemically generated, short ribonucleic acid (RNA), deoxyribonucleic acid (DNA) oligonucleotides or peptides that, through their 3-dimensional structures, bind to specific target molecules with high affinity and specificity [2]. Aptamers can be reengineered to undergo a conformational change upon binding a specific analyte and E-ABs can utilise this conformational change to generate a measurable electrochemical signal [1].

A group at the University of California, Santa Barba details in one of their papers, real time measurement utilising an E-AB sensing platform, in which the binding-induced folding of an electrode-bound, redox-reporter-modified aptamer, leads to a change in the rate of electron transfer, which is easily detected [1]. The paper highlights the capacity of detection of new molecules via the simple replacement of the aptamer recognition element on the platform. Through this, the group have fabricated sensors for recognising aminoglycoside antibiotics, inclusive of tobramycin, measuring *in vivo* concentrations following intravenous or intramuscular injections in rats [1]. The work documented in this report is to support validation of these biosensor measurements.

SECTION ONE

LITERATURE REVIEW: AMINOGLYCOSIDES

1.1 Introduction

1.1.1 Bacterial Infections

Bacterial infections are the proliferation of a harmful strain of bacteria on or inside the body, and each year in the US at least 23,000 people die from disease caused by bacteria. Pathogenic microorganisms are a known cause for diseases such as pneumonia, diarrhoea and meningitis, as well as blood infections. Many bacterial infections can be treated successfully with appropriate antibiotics, however resistance to antibiotics is now widespread among bacteria and is increasing at a disturbing rate [3]. This increasing frequency of bacterial strains that are resistant to available antibiotics demands the discovery of new therapeutic approaches.

Bacteria are single-celled microorganisms known as prokaryotes which utilise several mechanisms to cause disease in human hosts [4]. The pathogenesis, or the ability to cause disease, of bacterial infection includes initiation of the infectious process, through encountering a human host, and the mechanisms that lead to the development of signs and symptoms of disease. This is caused by the ability of a bacterial pathogen to illicit numerous host responses and mechanisms to evade the host defences [4]. Characteristics of bacteria that are pathogens include transmissibility, adherence to host cells, invasion of host cells and tissues, being toxigenic and persistence, and the ability to evade or survive the host's immune system.

Bacterial infections can occur through transmission of infection by several routes such as contact, vectors, vehicles and airborne transmission. These are examples of horizontal transmission, which is when organisms are transmitted from living person to living person. Pathogens can spread via direct contact with infected skin, mucous membranes, or body fluids as well as through indirect contact from those infected, contaminating surfaces. In this case, transmission occurs from strains of bacteria with the ability to survive outside of a host and remain infective for periods of time. Polluted food or water can act as common vehicles to spread pathogens alongside vectors, such as mosquitos transmitting disease. Airborne transmission can arise from long-term suspension of evaporated droplets or dust particles containing microorganisms.

Bacteria have a cell structure which is distinct from human cells, promoting the design of therapeutics with bacterial specificity. There are four major sites in the bacterial cell that allow antimicrobial targeting.

1) Cell wall (disrupts synthesis of cell wall).
2) The bacterial ribosome (disrupts protein synthesis)
3) The nucleic acid synthetic pathway
4) The cell membrane (disrupts cell membrane)

A key to fighting bacterial disease is the identification of these distinctive characteristics and ensuring appropriate treatment strategies are initiated early.

1.1.2 Aminoglycosides

Aminoglycosides (AGs) are broad-spectrum antibiotics (ABs) that are used against Gram-positive and Gram-negative bacteria [5], and are one of the oldest antibiotics [6]. They were first discovered in 1944 as molecules naturally produced by bacteria [6]. They are produced by many species of *Streptomyces*, deriving the suffix "mycin", or *Micromonospora*, giving the suffix "micin", bacteria [5].

The first aminoglycoside, streptomycin, was discovered by Waksman and co-workers, Albert Schatz, and Elizabeth Bugie, who isolated the compound from Streptomyces griseus, a strain of soil bacteria [7]. Since then Streptomycin has been found to inhibit the growth of a variety of bacterial organisms, including the organism that causes tuberculosis. A range of natural aminoglycosides, such as gentamicin and tobramycin, and semisynthetic aminoglycosides (synthetically modified AGs to generate those with extended activities), such as netilmicin and amikacin, have been identified and developed since then.

AGs are commonly used for treatment and prevention of infection and disease in both humans and animals, however, they poorly penetrate biological membranes [6]. Due to poor systemic absorption of AGs linked to their polarity, administration is usually via injection or the topical route [5], and they are excreted almost unchanged in the urine [6]. Currently, there are multiple formulations available on the market, including intravenous (i.v.) infusion, intramuscular (i.m.) and ophthalmic [8].

1.1.2.1 Mechanism of Action (MOA)

AGs are bactericidal and their mechanistic action is conducted through interfering with various aspects of protein synthesis [9] of oxygen dependent bacterial cells. Their activity results from binding to the surface of the bacteria and being transported through the cell wall [7], a proposed energy requiring, three step process explained below.

1) Energy Independent Step I – The first step is electrostatic surface binding, an energy independent binding of positively charged AGs to negatively charged moieties of lipopolysaccharides, phospholipids and outer membrane proteins [10]. This interference disturbs membrane function and is believed to result in the displacement of Mg^{2+} and Ca^{2+} ions that link adjacent lipopolysaccharide molecules [7]. This process damages the outer membrane and enhances its permeability [10]. As essential cell constituents leak out of the membrane, this results in cell death [7].

2) Energy Dependent Phase I – A threshold ion transfer potential is generated by a membrane-bound respiratory chain, utilising membrane ion transport systems [10]. This energy requirement explains the lack of activity of AGs in anaerobic environments [11], due to their low level of transmembrane potential. Energy dependent phase I leads to the uptake of AGs and binding to ribosomes, explained in Fig. 1.1. This triggers a series of events via loss of membrane integrity [10].

3) Energy Dependent Phase II – Additional AGs are transported across the damaged membrane, resulting in accumulation of AGs irreversibly saturating ribosomes and subjecting the cell to death [10].

Once inside the cell AG bactericidal activity is their ability to permanently bind to the decoding region of the 16S ribosomal RNA (rRNA) component of the 30S subunit of bacterial ribosomes [9], as shown in Fig. 1.1. The 30S ribosomal subunit plays a crucial role in the translation of genetic material [10]. Therefore, this results in impairing the proofreading process in the rRNA and the production of “defective proteins” within the cell membrane, leading to altered permeability [6]. Harold Neu in his paper gives the opinion that misreading of polypeptide synthesis occurs *in vitro*, and the major *in vivo* action of AGs structurally related to tobramycin, is perhaps related to depletion of the ribosome pool from irreversible binding to the ribosomal subunit [7].

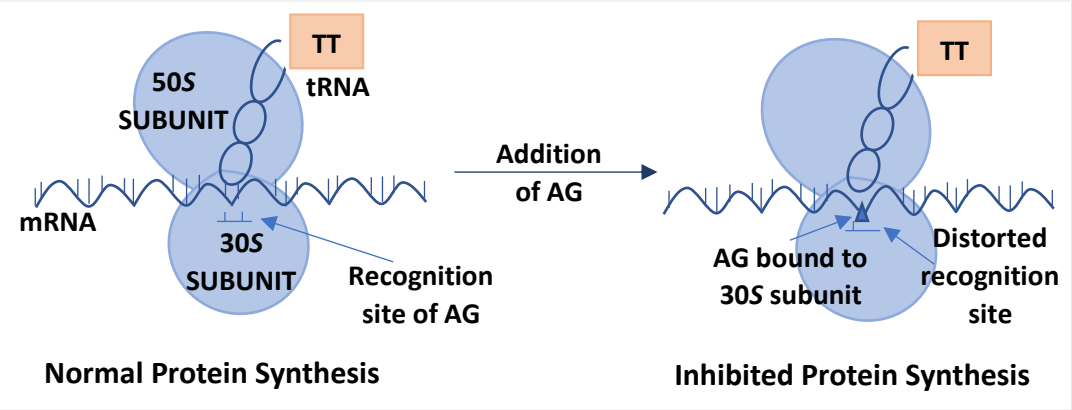


Figure 1.1: *Representation of protein synthesis inhibition by addition of AG. The AG blocks the initiation complex, leading to the mRNA being misread and the protein is incorrect. Image adapted from http://www.biologydiscussion.com/medical-microbiology/streptomycin-discovery-structure-and-mechanism-antibiotics/55919*

The pharmacodynamic characteristics of concentration-dependent killing of bacteria and post-antibiotic effect (PAE) have the potential to maximise the activity of AGs [6]. Drug concentration has a direct link with the bactericidal effect of AGs, with increasing toxicity as the concentration of antibiotic increases [10]. With post-antibiotic ability, AGs continue to exhibit bacteria killing effects at concentrations below the minimum inhibitory level, with extended duration as C_{max} increases [9]. Bactericidal effect can be prolonged by 0.5 – 7.5h, despite the absence of antibiotic from serum [6]. These two characteristics are reason to implementation of once daily dosing of AGs [6] in the clinic, which in turn is a method for reducing their association with nephrotoxicity.

1.1.3 Bacterial Resistance & Challenges

Inherent resistance to ABs has been in existence prior to the discovery of ABs as pharmaceutical agents. Bacteria can have inherent resistant against certain antimicrobial agents, or the ability to acquire resistance. Bacterial resistance can be defined as the ability of a microorganism to withstand the effects of a drug that are lethal to most members of its bacterial species, ultimately eliminating the effectiveness of the drug. This can be facilitated by mutation of their own genes or by the acquisition of extrinsic genes.

In recent years, antimicrobial resistance (AMR) has emerged as a global health problem [9], with high levels of bacterial resistance worldwide bringing AGs much attention [6]. Acquired resistance has occurred from extensive use of AGs in animal species therapy for infections of the respiratory, urinary and digestive tract, alongside human medicinal use. AG resistance can be caused by decreased antibiotic uptake and accumulation from reduced permeability of the cell membrane, modification of the ribosomal target, enzymatic modification of aminoglycosides, and efflux enhancement [10]. The most prevalent resistance to AGs in both Gram-negative and positive bacteria is caused by bacterial inactivation by intracellular enzymes.

Aminoglycoside modifying enzymes (AME) render AGs incapable of binding to its ribosomal target. Located in chromosomes or as part of the genetic material of bacteria, they alter the functional group structure of AGs. There are three types of AMEs, differing according to the action they exert,

1) Aminoglycoside nucleotidyltransferases (ANT`s) which transfer a nucleotide triphosphate moiety to a hydroxyl group
2) Aminoglycoside acetyltransferases (AAC`s) which transfer the acetyl group from acetyl-CoA to an amino group
3) Aminoglycoside phosphotransferases (APH`s) which transfer the phosphoryl group from ATP to a hydroxyl group

These are then subdivided according to their exact target site on the AG [6]. With over a hundred different AMEs, resistance is not easy to predict from one AG to another and with the insurgence of resistance often combination therapy may be used. The clinical use of AGs such as penicillin and streptomycin in the 1960s and 1970s, resulted in this emergence of drug-resistant bacteria to these antibiotic agents, leading to serious infections. Many strategies have been elicited to overcome AMEs, such as semisynthetic AGs, altering the target for AMEs. In the 1970s, development of AG derivates resulted in semisynthetic AGs advanced to overcome the problems related to drug resistance and toxicity [9], these include amikacin (derived from kanamycin) and netilmicin (derived from sisomicin), currently being marketed as chemotherapeutic agents against resistant bacteria. Despite emerging overall antimicrobial resistance, AGs retain their broad spectrum of activity, with current treatment options described in the following section.

1.1.4 Current treatments

Since the discovery of streptomycin, various AGs have been established and introduced into the clinic for infectious disease. Streptomycin was the first drug used effectively against tuberculosis [6], and currently AGs remain valuable against various infections in humans and animals.

The advantages of AGs which support their clinical use are broad antibacterial spectrum targeting both Gram-positive and Gram-negative bacteria [5], rapid bactericidal action (Fig. 1.1), chemical stability without metabolic changes and the wide-ranging efficacy and success, gained from use over many years [7]. However, alongside the advantages are some drawbacks of known nephrotoxicity and ototoxicity, variable pharmacokinetics and reported lack of correlation between the administered dose and measured serum concentrations [7].

AGs were topically used for the definitive treatment of several diseases, including plague and brucellosis [6] in the early days of antimicrobial chemotherapy. In veterinary care, commonly used AG examples are neomycin, dihydrostreptomycin and spectinomycin targeted against infections of the digestive tract in many animal species including cattle, pigs, poultry, horses, dogs and cats. In humans, AGs gentamicin, tobramycin and amikacin are applied systemically for infections involving multidrug-resistant Gram-negative bacteria, such as *Pseudomonas, Acinetobacter,* and *Enterobacter*. Further examples of AG use, with respect to route of administration, are given in Table 1.1.

Table 1.1: *Considerations for the use of aminoglycosides (adapted from [6])*

Application	Aminoglycoside	Use
Topical	Neomycin, gentamicin and tobramycin	Infections of the eye and outer ear
	Gentamicin	Cutaneous leishmaniasis
Oral	Neomycin and erythromycin	Colorectal surgery prophylaxis
	Paromomycin	Protozoan infections

Combination Therapy

Siegenthaler *et al*. [7] state that reviews of many studies in which AGs have been used as monotherapy reveals success rates of 24 to 100 percent [7]. Monotherapy is usually enlisted for treatment if less serious infections such as urinary tract infections, or when other ABs are not appropriate for use [12]. For specific targeted therapies AGs are often given in combination because of the ability to broaden the spectrum of antibiotic therapy by,

1) Enhancement of antibacterial activity due to synergistic or additive interactions
2) Reduction in dosage regimens and in consequent toxicity related side effects
3) Reduction in the risk of therapeutic failure because of bacterial resistance

The effect of two antimicrobial agents is greater than the sum of their individual effects, a well characterised effect between AGs and cell-wall-active agents, such as beta-lactams in both Gram-negative and positive organisms [13]. The synergism demonstrated when AGs are used alongside beta-lactam ABs, continues to be the optimal therapy [7] as they are synergistic against streptococci enterococci, *Pseudomonas* and other gram-negative bacteria [14]. This combination of β-lactam–aminoglycoside therapy, allows for the interaction of two different mechanisms of bacterial killing with β-Lactam-mediated disturbance of the cell wall, facilitating the passage of AGs into the periplasmic space to bind to ribosomal DNA (Fig. 1.1) [15]. The AG tobramycin displays its main role in combination with β-lactam agents against gram-negative pathogens of cystic fibrosis and *Pseudomonas endocarditis* [16].

1.1.4.1 Beta-lactam antibiotics (β-lactam)

β-Lactams are one of the most widely used class of ABs, sharing a common structure and mechanism of action (MOA). They include penicillin, cephalosporins, and carbapenems, all containing a 4-membered beta lactam ring fused to a second ring, essential for antibacterial activity, inhibiting cell-wall synthesis [17]. Their basic MOA involves the interruption of the transpeptidation process vital for linking the individual peptidoglycan components of the bacterial cell wall together [17]. Basic elements of the MOA have been understood, however the complete process is yet to be discovered [17].

Fig. 1.2 illustrates the MOA of β-Lactam agents. In the absence of drug, Penicillin Binding Proteins (PBPs) work to catalyse cross-links between adjacent glycan chains in the cell wall,

which involves the removal of a terminal D-alanine residue from one of the peptidoglycan chains [18]. PBPs are enzymes (transpeptidases, carboxypeptidases, and endopeptidases) with key function in reshaping the cell wall during growth and division [17]. Glycosyltransferases (GT), which exist as either separate subunits, or tightly associated with transpeptidases, create covalent bonds between adjacent sugar molecules N-acetyl-muramic acid (NAM) and N-acetyl-glucosamine (NAG), resulting in a rigid cell wall. The cell wall protects the bacterial cell from osmotic forces that would otherwise result in cell rupture [18].

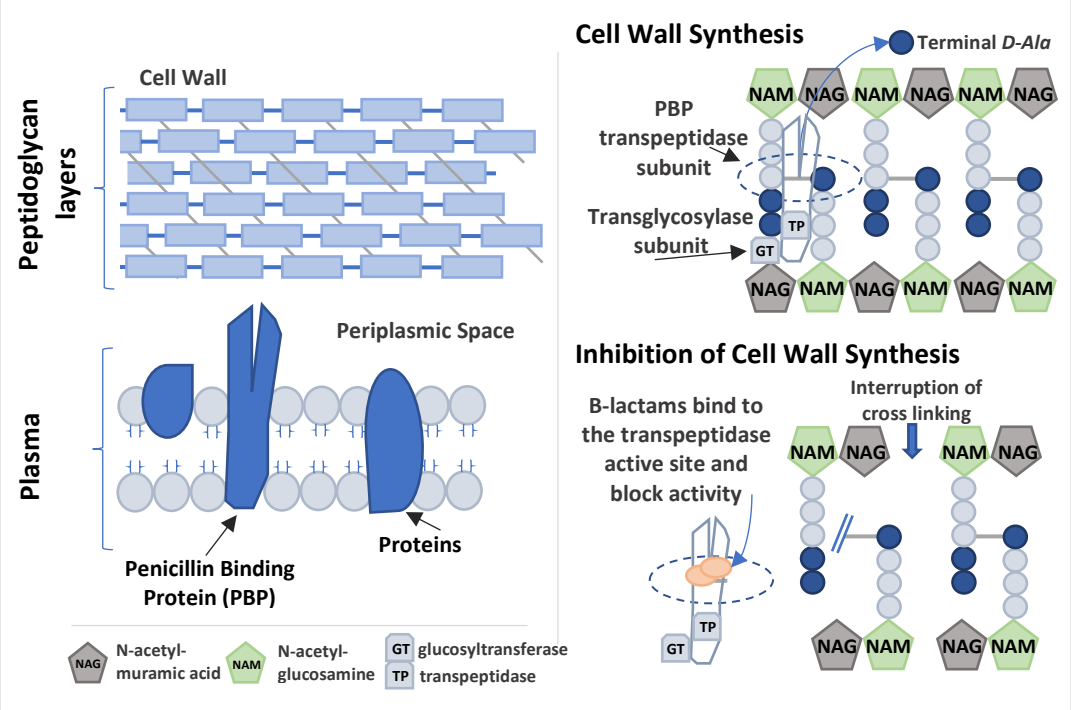


Figure 1.2: *Representation of β-lactam antibiotic mechanism of action.*

Image adapted from [18].

β-Lactam ABs bind to and inactivate PBPs, located on the inner surface of the bacterial cell wall [17]. The ABs, which include penicillin, cephalosporins, monobactams and carbapenems, bear a structural resemblance to the natural D-Ala-D-Ala substrate for the transpeptidase and exert their inhibitory effects on cell wall synthesis, by tightly binding to the active site [18]. β-Lactam ABs have differing affinities for the various PBPs and therefore depending on the specific PBP bound, they exert a different effect upon the bacteria [17].

Like AGs, bacterial resistance exists too for β-lactams, with the production of beta-lactamases by bacteria, working to inactive the antibiotic through hydrolysis of the cyclic amide bond of the beta-lactam ring [17]. Knowing this, research has been conducted to produce biochemical modifications to decrease susceptibility to β-lactamases [17].

β-lactam agents are favourable since they have an approving ratio of therapeutic to toxic effects alongside promising PK characteristics [17]. Therapy as single agents has been limited, therefore for serious infections these agents are usually used in combination therapy. The synergism demonstrated when AGs are used alongside β-lactam ABs, continues to be the optimal therapy [7] as they are synergistic against streptococci enterococci, *Pseudomonas* and other gram-negative bacteria [14]. This combination of β-lactam–aminoglycoside therapy, allows for the interaction of two different mechanisms of bacterial killing with β-Lactam-mediated disturbance of the cell wall, facilitating the passage of AGs into the periplasmic space to bind to ribosomal DNA (Fig. 1.1) [15]. The AG tobramycin displays its main role in combination with β-lactam agents against gram-negative pathogens of cystic fibrosis and *Pseudomonas endocarditis* [16].

1.1.4.2 Monitoring

Historically AGs were administered in multiple doses, every 8 - 12 hours [14], however as the PK and pharmacodynamics (PD) have been better understood, once daily dosing has become the standard of care [13].

Ototoxicity and nephrotoxicity are the most reported adverse reactions, with nephrotoxicity occurring in 5–10% of patients. Ototoxicity results from exposure to AGs via the blood system, damaging the inner ear or the vestibulocochlear nerve (involved in sending balance and hearing information from the inner ear to the brain). Nephrotoxicity occurs from AG accumulation in the proximal tubules post glomerular filtration, resulting in mitochondrial swelling and kidney damage. The variability of occurrence is dependent upon the AG used, the dose administered, the age of the patient, levels of dehydration, and the trough serum concentrations obtained. As nephrotoxicity involves the proximal tubules, this adverse effect may be reversible over time due to the capability of regeneration of the tubules [12].

Hammett-Stabler *et al.* [12] report that ototoxicity occurs in 0.5 to 3 % of patients, but some studies suggest the incidence may be higher and closer to 25% [12]. Unlike nephrotoxicity, this adverse reaction is not reversible with the potential for cochlear damage or vestibular impairment. As the relationship between toxicity and serum concentration is apparent, monitoring of AGs is important. Being aware of the AG serum concentrations is important in preventing toxicity, however alongside it is key to monitor the clinical symptoms such as hearing problems and vertigo, especially for those on prolonged duration [6].

Monitoring includes tests of renal function in addition to serum drug concentrations to screen activity and toxicity. The method of monitoring differs according the administration method, for example with multiple daily regimens measuring a peak level 30 minutes after completion of the second or third dose and trough levels right before the next dose [6]. In extended interval dosing, clinicians can either measure a trough level 1 hour before the next dose, continuing if renal function is stable [6]. Given the low concentrations expected, it is recommended to test these samples immediately or store frozen prior to analysis [12].

1.1.5 Tobramycin

Tobramycin, produced by *Streptomyces tenebrarius*, is a cationic, water-soluble AG antibiotic widely used against Gram-negative bacterial infections [8, 19]. The general structure of AGs is characterized by two or more amino sugars linked by glycosidic bonds to an aminocyclitol scaffold [5]. The structure of Tobramycin is show below in Fig. 1.3.

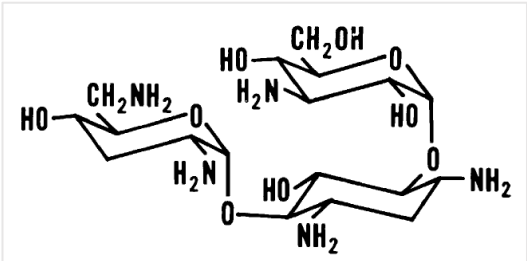


Figure 1.3: *Structure of Tobramycin* [11]

Tobramycin is composed of a 2,6-diamino2,6-dideoxy sugar and a 3-amino-3-deoxyglucose linked to the 4- and 6-hydroxyl groups of 2-deoxystreptamine [11]. Tobramycin is present as a white to off-white powder and has an empirical formula of $C_{18}H_{37}N_5O_9$ and a molecular

weight of 467.52 g/mol. It is water soluble, polar and stable at ambient temperatures at a wide range of pH 1 to 11 [20].

Tobramycin acts against gram negative bacteria much like all AGs by inhibiting protein synthesis through permanently binding to the 30s ribosomal unit [11], with greater activity against *Pseudomonas aeruginosa* [11]. Currently, therapeutically, Tobramycin is being used for infections in animals as well as in combination with a beta-lactam agent against Gram-negative bacterial pathogens [16]. A key drug for the management of Cystic Fibrosis (CF) because of its activity against *P. aeruginosa*, Tobramycin is administered via the intravenous and inhaled routes [16], in the European Union (EU) and United States (US). The inhaled delivery of AGs has become an area of wide interest because of the greater local exposure within the lungs with reduced systemic toxicity [13]. Tobramycin is also administered as treatment for the uncommon disease, *Pseudomonas endocarditis* (PsE) [16].

1.1.5.1 Quantitation Methods for Tobramycin and AGs

AGs are not only used for prevention of disease in humans but also for veterinary medicine and animal husbandry [21], however due to their toxicity and potential antibiotic resistance, substantial consideration is being paid to their associated risks. AGs have a narrow therapeutic window, with their pharmacokinetic properties varying from one person to another due to differences in tissue distribution and renal function of the patient [5]. For this reason, close monitoring of serum levels is important to assess potential toxic effects, of nephrotoxicity and ototoxicity, in the renally impaired, those on long-term therapy and with age-related variations in metabolism [5] [12].

It is essential to monitor AG concentration levels and dose-dependent side-effects for adequate treatment and avoidance of toxic effects [12]. However, quantitation of tobramycin and AGs are analytically challenging due to their physio-chemical properties, hydrophilic structures and lack of strong chromophores in their molecule structures [8, 19]. The structure of tobramycin, shown in Fig. 1.3 indicates that tobramycin has five primary amines, one primary OH group and four secondary OH groups. Their structure deems them highly polar due to various amino and hydroxyl groups and they are known to create strong links with matrix proteins [22], leading to high background signals, impacting the signal: noise (S:N), sensitivity and selectivity of methods. The S:N allows quantification of how prominent

the analyte of interest is, in comparison to the background signal from all the other species present.

As tobramycin usage increases for the treatment of serious gram-negative bacillary infections, there is a need for a simple, rapid, selective, and sensitive method for analysis [8], and monitoring of trace level residues in complex matrices [21]. Current methodologies try to overcome these structure-related challenges but also have limitations**.** Determination of AGs using conventional HPLC with spectroscopic UV detection is difficult because of this lack of UV chromophore. Therefore, traditional methods for detection include bioassays, GC–MS, HPLC, introduction of a chromophore group onto the molecule via derivatisation, as well as immunoassay and spectrophotometric methods [8, 23].

The use of derivatizing agents to improve detection and/or separation by ultraviolet absorbance (UV) or by fluorescence [24] can often be time consuming, and introduce impurities, working against rapid quantification. With derivatisation there is the possibility of self-degradation of the derivatives formed, leading to poor reproducibility caused by their instability. Immunoassay for tobramycin determination can too be time consuming, as well as expensive with the need of specific reagents. Therefore, these methods are effective, however are limited by time, complicated workflows, selectivity and often the requirement of specialised equipment [12][5].

Electrochemical analysis is being viewed increasingly as an alternative detection method [8], as well as LC-MS/MS due to advancements in detection technologies in liquid chromatography and tandem mass spectrometry [5]. MS being a very sensitive and selective analytical technique is an attractive choice to monitor low ng/mL range of AGs in samples, without the need for derivatization. The advantages of this technique for Tobramycin detection will be explained in the following sections.

SECTION TWO

TOBRAMYCIN LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY (LC-MS/MS) METHOD DEVELOPMENT

2.1 LC-MS/MS

2.1.1 Overview of LC-MS/MS

High-performance liquid chromatographic (HPLC) in combination with tandem mass spectrometry (MS/MS) is a common tool used for the quantification of an analyte of interest in a biological fluid. The technique involves the separation of the analyte from other endogenous substances in the biological sample by liquid chromatography, followed by the ionisation of this analyte of interest. The ions produced are introduced into the mass spectrometer, where they are analysed based on their mass-to-charge (*m/z)* ratios via an array of electric and magnetic force fields [25]. The ions are detected, and their relative abundances are recorded. The use of liquid chromatography tandem mass spectrometry (LC-MS/MS) has become the analytical technique of choice in drug discovery and development [25], reducing timelines for method development whilst increasing sensitivity for quantification of analytes from complex biological matrices [26]. Literature presents examples for tobramycin analysis via LC-MS/MS, holding the advantages of sensitivity and selectivity for low levels of Tobramycin in complex matrices.

2.1.1.1 Liquid Chromatography

Chromatography is a broadly used technique to separate a mixture of two or more analytes based on differences in their polarity. The word “chromatography” literally means “colour writing” and was first recognised by Russian botanist, Tswett, who used a simple form of liquid-solid chromatography to separate several plant pigments [27]. Today, the technique has little to do with separation based on colour and is used to refer to all chromatographic separation involving a mobile phase and a stationary phase.

Liquid chromatography refers to separation occurring through intermolecular competition between the stationary phase and the mobile phase for the components of the analyte mixture. There are several types of liquid chromatography differing by the physical state (gas or liquid) of the mobile phases and stationary phases. Within this report, the focus shall be on liquid-solid chromatography, indicating a solid stationary phase and a liquid mobile phase.

The stationary phase in column chromatography does not move and is most typically a fine adsorbent solid; vital for efficient separation of components in a mixture. The activity of the adsorbent is represented by the measure of an adsorbent's attraction for solutes in the sample solution. The most common stationary phase is extremely fine particles of silica (SiO_2), a solid with an extended structure of tetrahedral silica atoms bridged together by bent oxygen atoms. The small particle size of silica produces a large surface area that strongly adsorbs molecules via intermolecular forces such as hydrogen-bonding and dipole-dipole interactions. The surface of the silica particles consists of very polar silanol (Si-O-H) groups, therefore the more polar an analyte is, the more strongly it adheres to the silica stationary phase, resulting in slower movement through the stationary phase.

The mobile phase consists of eluents of differing polarities, which move through the stationary phase to separate analytes. As the polarity of the mobile phase increases, dependent upon the identity of the organic solvents used, the faster analytes will move through the stationary phase. This is due to the polar mobile phases being a stronger competitor than the analyte molecules to the stationary phase. Beginning elution with a solvent of lower polarity is recommended to elute those analytes with weak adsorption for the stationary phase, followed by changes in gradient or solvent, altering the polarity to elute all analytes over time.

2.1.1.2 Normal Phase and Reverse Phase Chromatography

The physical separation technique of HPLC follows the above, where each of the components (sample and mobile phase) have differing degrees of integration with the stationary phase, altering the rate at which they travel through the column and elute. There are several modes of HPLC that can be used depending on the relative polarity of the stationary and mobile phases. When the polarity of the column used for separation is greater than that of the mobile phase, this is defined as normal phase chromatography. Polar analytes will adsorb to the stationary phase, with less polar analytes eluting faster, therefore elution will be based on order of increasing polarity.

Reverse phase (RP) chromatography is the reverse of normal phase, whereby polar hydrophilic mobile phases (e.g. water, methanol, and Acetonitrile) are used against non-polar hydrophobic stationary phases. Coupled with gradient elution changing the mobile phase composition over time, analytes with a degree of hydrophobic character within the

solution will move and elute at different speeds with excellent recovery and resolution [28]. Reverse phase chromatography is favoured for MS as the mobile phase composition can be tailored to suit ionisation. In bioanalysis, due to the non-polar nature of drug analytes, with the majority being required to pass through several cell membranes to reach the intended target, RP chromatography is often the main HPLC mode used. Silica particles modified with octadecyl (C18) groups and a water/Acetonitrile solution are examples of some of the most common stationary and mobile phases used in reverse phase HPLC.

In RP HPLC, water can be considered as one of the weakest solvents as it is the most polar. Therefore, hydrophobic, non-polar analytes will remain in the stationary phase for longer with this solvent, extending retention times. Organic solvents are added, and as they are less polar, the (hydrophobic) analyte is no longer as strongly repelled into the stationary phase, leading to earlier elution. This makes the modifier chromatographically 'strong' as it can speed up elution and reduce retention. As progressively more organic modifier is added to the mobile phase, the analyte retention time will continue to decrease.

2.1.1.3 Isocratic and Gradient Elution

Elution chromatography in HPLC can be carried out in two ways, either isocratic or gradient mode [29]. When the composition of the mobile phase is held constant throughout the sample analysis, it is known as isocratic elution. In this mode, sample retention can be controlled by varying the composition of the single mobile phase, being a mixture of a 'weak' solvent A and a 'strong' solvent B. Isocratic elution is known to be susceptible to issues of band broadening and longer analysis times.

Gradient elution in RP refers to where the composition of the mobile phase is varied throughout analysis, usually with an increase in organic solvent percentage e.g. 0 to 100%v ACN – water [29]. Generally gradient modes are useful for separation of samples containing a mixture of analytes with differences in polarity, thus not being ideal for separation via a single mobile phase. In bioanalysis, biological samples often contain additional strongly retained components, alongside the analyte of interest. Therefore, during isocratic elution, repeated injection can lead to accumulation of these components on the column with follow on effects of loss of column efficiency and changes in retention [29]. Gradient elution allows for cleansing of the column with a strong solvent (100% mobile phase B) at the end

of the gradient, helping to reduce any accumulation of contaminating components on the column [29].

There are many variables that can dictate the success of the chromatographic separation, including stability of the samples, cleanliness of the material to be injected, LC separation, chromatographic resolution and mass resolution. Specifically, for Tobramycin, due to its hydrophilic properties, as with AGs, RP-chromatography does not provide adequate separation alone, and therefore methods with mobile phase containing counter-ions reagents, forming ion pairs, have been widely used [24]. There are several key considerations for successful chromatography, which will be discussed in the following section.

2.1.2 Chromatographic Considerations

2.1.2.1 Columns

To maximise the output from RP-chromatography, many parameters need to be optimised during method development, dependent upon the structure of the molecule. Selection of the stationary phase, mobile phase, and organic solvents can determine the robustness of quantification method. Selecting a high-performance, stable and reproducible column is important [28], to limit variability and ensure robustness of the LC method. Factors such as column chemistry, length, pore size and temperature are vital to consider during optimisation as well effects upon resolution, retention and efficiency, as explained below.

In RP-chromatography, the most common non-polar stationary phases use an organochlorosilane where the R group is an n-octyl (C_8) or n-octyldecyl (C_{18}) hydrocarbon chain [28]. Columns with C_{18} groups attached to the particles for reverse-phase interactions, are widely used in bioanalytical laboratories and generally suitable and recommended for all samples [25]. This hydrophobic column is used to retain non-polar compounds. It is densely packed to increase the interaction between the elute and stationary phase to ultimately advantage separation ability. In general, a C_8 alkyl chain column will shorten the retention time (RT), with only slight differences in separation with a C_{18}.

HPLC and ultra-high-performance (UHPLC) liquid chromatography represent the most commonly applied liquid chromatography techniques today. UHPLC was developed from HPLC by improvements of the separation process efficiency and adjustments of the instrumentation. The efficiency of chromatographic separation can be described by the Van

Deemter equation (Section 2.1.2.4), reflecting that smaller particle sizes ensure better mass transfer and minimal band broadening. This led to the development of columns with sub-2 µm particles, with higher selectivity and sensitivity.

Throughout this project, two specific types of column were used for separation, the Waters C18 column range and the Phenomenex Synergi™ columns. Both are suitable columns for tobramycin separation and are explained in detail below.

2.1.2.1.1 Waters Acquity™ UPLC C18 Bridged Ethylene Hybrid (BEH)

The UPLC columns currently being produced by Waters are specifically designed for use with their Acquity™ UPLC systems, which was the chosen LC system for this project. These columns are packed with particles 1.7 µm in diameter, compared to conventional columns with particle sizes of 3 to 5 µm and possess a bridged ethylene/silica hybrid (BEH) structure, shown in Fig. 2.1. The structure of the BEH particle has shown improved efficiency, strength and pH range (pH 1-12), enhancing the column's mechanical stability. This allows for operation at higher pressures and temperatures.

Acquity™ C18 BEH columns are universal columns since they can be used for molecules suitable for RP separations. The C18 stationary phase is one of the most commonly used stationary phases in UPLC columns, providing a starting point for method development. The small size of the particles results in the columns producing a very high backpressure on the system, that conventional HPLC systems do not have the capability to handle, however the Acquity™ system can deal with this.

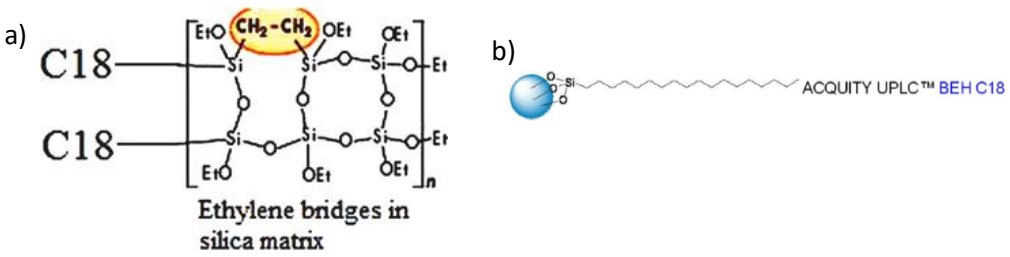


Figure 2.1: *a) Structural chemistry of a BEH C18 column [30], b) Acquity™ C18 BEH columns [31]*

2.1.2.1.2 Waters Acquity™ UPLC C18 Charged Surface Hybrid (CSH)

The CSH column is based on the Waters organosilica BEH, fully porous particle technology, however with the added advantage of a low-level charge from basic moieties [32]. This improves sample loading capacity and peak asymmetry, whilst maintain the mechanical and chemical stability provided by the BEH. As tobramycin is a very basic compound, the CSH column is ideal for retention of basic polar compounds under low pH.

2.1.2.1.3 Phenomenex Synergi™, RP Columns

The polar-RP column is an ether linked phenyl column and is polar endcapped. It offers advantages of retention and selectivity for highly polar compounds with high levels of reproducibility. Alongside this column, the hydro-RP polar endcapped column offers improved polar selectivity. Extremely polar analytes are not always retained and often do not separate well on conventional C18 columns; therefore the Hydro-RP is a C18 bonded phase, endcapped with a unique proprietary polar group to provide extreme retention of both hydrophobic as well as polar compounds under 100 % aqueous conditions. Through a combination of greater retention, excellent efficiency, and stability it is ideal for MS applications.

As well as column chemistry, table 1.2 provides an overview of other critical factors to consider during method optimisation of RP-chromatography.

Table 2.1: *Critical parameters in reversed phase chromatography*

Factor	Effect
Column Length	Longer columns provide better separation due to higher theoretical plate numbers [28]. Smaller organic molecules are more sensitive to changes in column length than high molecular weight biomolecules.
Pore Size	The size of the pores has an important impact upon the accessibility of the analyte for the stationary phase. It has a key role in determining the resolution and achieving optimal separation.
Temperature	Impacts viscosity, as increased temperature, reduces viscosity, leading to improved peak shape and decreased run time [33].
Flow Rate	A principal factor for resolution of small molecules, affecting longitudinal diffusion of the solute molecules.

2.1.2.2 Retention and Resolution

Retention factor (k) is a term often used in chromatography and refers to the ratio of the amount of time an analyte is bound to the stationary relative to the amount of time it is bound to the mobile phase. Fig. 2.2 shows the equation used to calculate k from t_R (retention time of analyte) and t_M (retention time of mobile phase).

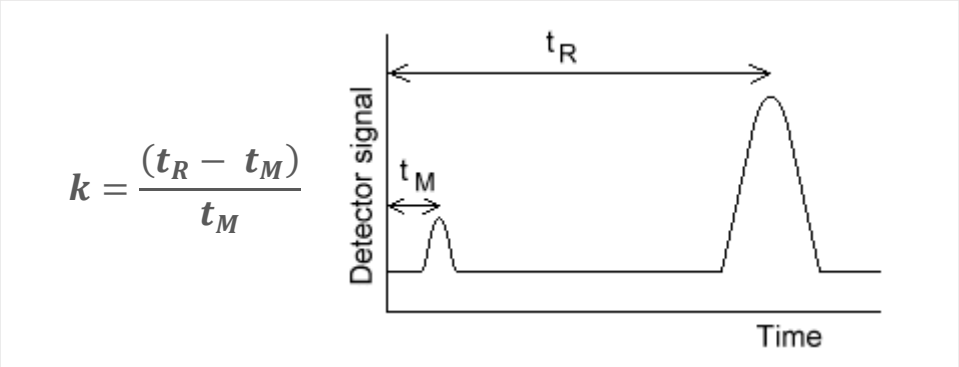


Figure 2.2: *Schematic illustrating how a retention factor can be calculated from a chromatogram.*

Image taken from www.shu.ac.uk/schools/sci/chem/tutorials/chrom/chrom1.htm.

The retention factor is useful when developing a method for detecting and quantifying an analyte, as it can provide an estimated retention time for when the analyte should be accurately quantifiable. Accurate quantitation is achieved when k ≥ 2 as this has given the analyte enough time to be resolved from the mobile phase. The RT can be used to precisely identify an analyte, as it will be constant for specified conditions. In addition, RT can help improve sensitivity as the MS can be instructed to look for different ions in different time periods.

Selectivity can be defined as the ability of the system to chemically distinguish between sample components in a mixture. Each component will have different degrees of interaction with the stationary phase, changing with factors such as organic solvent strength, nature and composition of the mobile phase, mobile phase pH, gradient shape, surface chemistry of the stationary phase and temperature.

Resolution (Rs) is a measure of the degree of separation, determined from the difference in retention times of two adjacent solutes in a mixture. In chromatography, the peak profiles

are normally assumed to be Gaussian, and for accurate quantification, it is essential that two adjacent peaks be well resolved from one another.

The most common equation for measuring resolution (Rs) is given in equation 2.1 [34]:

$$Rs = 2\frac{(t_2 - t_1)}{(w_1 - w_2)}$$

(Equation 2.1)

Where t_2 and t_1 are the retention times of the two peaks of interest, and w_1 and w_2 are the corresponding peak widths measured at the baseline [34]. A value for Rs greater than one demonstrates complete separation of peaks, whereas a value below one eludes to peak overlapping. The three chromatographic variables: efficiency (N), selectivity and retention (k) can all affect resolution.

2.1.2.3 LogP

The hydrophobicity of a compound can give an indication to the level of retention that will be observed in RP-HPLC. Hydrophobicity can be expressed as LogP and shows the partition ratio of a compound between two immiscible solvents, usually octanol and water, under standard conditions (Equation 2.2) [35]. The partition coefficient (LogP) is the ratio of the concentration of the compound in octanol to its concentration in water.

$$\log P_{oct/wat} = \log\left(\frac{[analyte]_{octanol}}{[analyte]_{water}}\right)$$

(Equation 2.2)

A high value of LogP indicates a hydrophobic compound and a low value of Log P indicates a hydrophilic compound. Tobramycin has a LogP of approximately -7, indicating reflects its higher degree of hydrophilicity and its capability of more easily distributing into the aqueous phase. Tobramycin is by far mostly water soluble and is highly hydrophilic.

2.1.2.4 Efficiency, Van Deemter and Eddy Diffusion

The efficiency of a column is reported as the number of theoretical plates, N, a concept introduced by Martin and Synge in the 1940s, from experience with fractional distillation[36].

The number of theoretical plates is a measure of the "goodness" of the column and this related measure of system efficiency is the determined calculated using equation 2.3 below,

$$N = 16\left(\frac{t_r}{w}\right)^2$$

(Equation 2.3)

Where N is the number of theoretical plates, tr is the migration time of the analyte, i.e. the retention time from the beginning of injection and W is the width at the peak base, obtained by extrapolating tangents from the sides of the Gaussian curve to intercept the baseline. The distance between the intercepts is the peak width. A chromatogram with an early eluting peak (a low tr) that are broad in shape (large w), result in a small N value. On the other hand, a chromatogram with a late-appearing peak (large tr) and a very narrow peak shape (small w) will produce a large N. The higher the value of N, the greater the separation efficiency of the column. N can be affected by numerous variables including flow rate, stationary phase and temperature, all affecting peak broadening [36].

Theoretical plates are utilised in HPLC as a way of depicting separating efficiency. Separation occurs as an analyte passes through the chromatographic system, however the longer an analyte remains within the system the broader the resulting peaks become. This equates to band broadening, reducing the quality of separation, with consequences of poor peak shape, resolution and column efficiency.

Van Deemter published the most dominant study in relation to band broadening in 1956 [36], highlighting how eddy diffusion (the A term), longitudinal molecular diffusion (the B term), and mass transfer in the stationary liquid phase (the C term) contribute to band broadening. The equation in terms of column efficiency (H) in relation to the average linear velocity of the mobile phase (ν) is shown in equation 2.4.

$$H = A + \left(\frac{B}{v}\right) + Cv$$

(Equation 2.4)

Eddy diffusion (A) can be related to the fact that an analyte, within a 'band' of analytes, can take one of many pathways through the column. These multiple paths arise due to differences in column packing and small variations in the particle size of the packing material, in the stationary phase. The presence of multiple pathways effects the band of analytes as they move through the column, as can be seen in Fig. 2.3.

The average diameter of the analyte and the diameter of the particles of the stationary phase, have a direct influence upon the path taken by the analyte. A particle with a short pathway will elute ahead of one with a more complex pathway. Therefore, as shown in Fig. 2.3 particles two and three will elute after particle one. As the mobile phase flow rate is controlled via the HPLC it remains constant in the system. Band broadening occurs as analyte particles separate and adopt different paths through the band. Eddy diffusion can be minimised by selecting well packed columns utilising smaller stationary phase particles. This reduces variations in the distances between multiple routes through the chromatographic bed band [33].

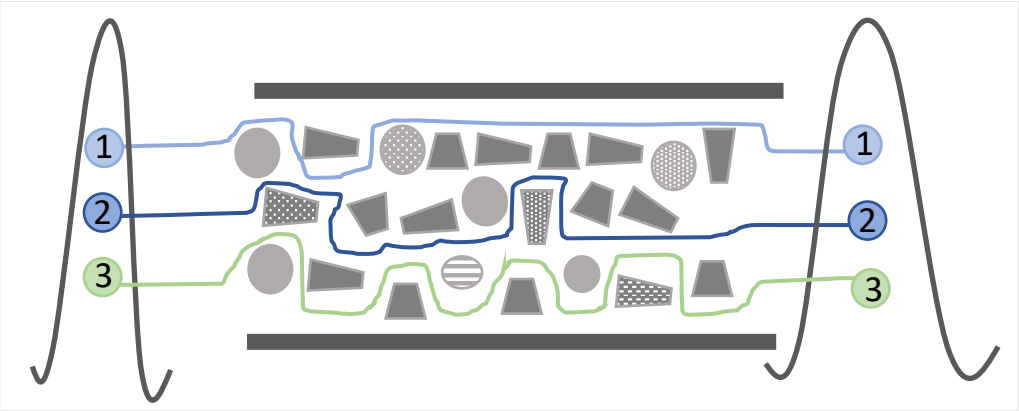


***Figure 2.3**: Schematic illustrating the different paths of three different molecules traveling through a packed bed of particles. Molecules enter the column at the same time and exit the column at different times because of the different paths they take.*

Longitudinal molecular diffusion (B) relates to the broadening factor occurring when an analyte band moves through the column and diffusion occurs from the higher concentration of the analyte at the band centre to the lower concentration at the band edges. Longitudinal diffusion has a much larger effect at low mobile phase velocity. As the analyte band is driven by mobile phase flow, longitudinal diffusion can be decreased by increasing the flow rate of the mobile phase with narrow columns. Reducing the time, the analyte spends within the column, reduces the time available for diffusion, ultimately minimising band broadening. For HPLC, high velocities are desirable to minimise analysis time for high throughput, therefore molecular diffusion is often of little interest in troubleshooting band broadening [36].

Mass transfer (C) essentially accounts for the time taken for the equilibrium between the mobile phase and stationary phase to be established. As the stationary phase material is porous, analytes within the mobile phase move quicker than those within the stationary phase, via diffusion. Broadening of the band results from the mass transfer of the analyte into and out of the stationary phase. The porous stationary phase creates a very large surface area for separation to occur, and the analyte molecules are diffusing into and out of these pores, some penetrating more deeply into the pore and becoming trapped. As the mobile phase continues to move forward at the same rate, this increases the band width. Mass transfer can be minimised by using smaller stationary phase particles, lower mobile phase flow rates, and heating the column to speed up the diffusion processes.

2.1.2.5 pH and pK_a

Chromatography is based upon the distribution, or partition, of analytes between the mobile phase and the stationary phase. The equilibrium of analytes between two distinct phases, referred to as the distribution or partition coefficient, k_d, is represented by equation 2.5,

$$\boldsymbol{K_d\ (or\ P) = \frac{[stationary\ phase]}{[mobile\ phase]}}$$

(Equation 2.5)

In RP-chromatography, the stationary phase and the mobile phase are part of a dynamic system, as there is a continuous flow of mobile phase over the column. This uninterrupted dynamic results in an equilibrium never being reached and ensures that analytes are transported along the column. The continuous dynamic is maintained, with some proportion of analytes interacting with the stationary phase and the remaining analytes moving through the column in the mobile phase.

The level of interaction is determined by the affinity of the analyte for the stationary phase. A high k_d value denotes that there are strong interactions with the stationary phase, and not much affinity for the mobile phase, resulting in no movement through the column. A low k_d value is the vice versa of the above and outcome is rapid movement through the column.

A key factor in liquid chromatography is the pH of the mobile phase, aiming to aid successful chromatographic separation of analytes in solution, through regulating the ionisation characteristics [28]. Mobile phase solutions usually contain strong acids at low pH and large volumes of organic solvents. Therefore, ionisation capability will depend on their pH [28].

In RP-chromatography successful suppression of analyte ionisation leads to improvements in peak shape and retention on the column. Based on the *pKa* of a compound, an appropriate mobile phase can be selected. A rule of thumb is selecting a buffering system with a range of +/- 2 pH units from the *pKa*, at a concentration with enough buffering capacity. An acidic compound reaches ionisation state at a pH > *pKa*, therefore if presented with a pH < *pKa*, the acidic compound will have greater retention. On the other side, a basic compound is protonated if the mobile phase has a pH < *pKa*. Consequently, for RP-Chromatography, retention is increased as pH increases above the *pKa*.

2.1.2.6 Ion pairing

Retention of compounds in RP-chromatography can be modified by mobile phase pH, as mentioned above. Additionally, an ion-pair reagent (IPR) can be added to the mobile phase to increase retention of an analyte. An IPR is a molecule containing a large hydrophobic moiety, which works by interacting with the stationary phase, and an ionic site with a charge opposite to that of the analyte of interest [5] (Fig 2.4).

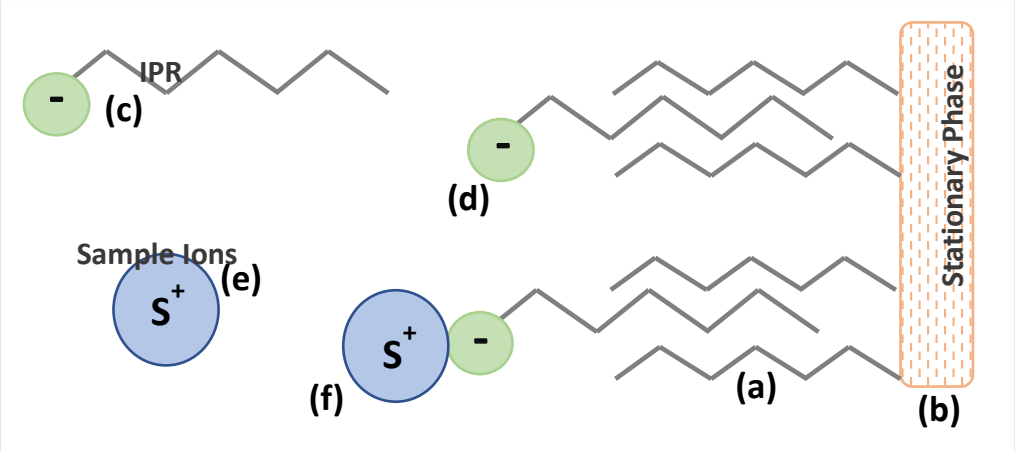


Figure 2.4: *Schematic illustrating of RP-IPC. Image adapted from [37].*

The RP-IPC is bonded (a) and attached to silica particles (b) in the stationary phase. The IPR (c) is dissolved into the mobile phase and adsorbed to the column (d). The sample ions could be either free and unretained (e) or retained and separated on the column (f), by the IPC.

The hydrophilic properties of aminoglycosides often result in poor separation by RP-chromatography, therefore mobile phases containing ion pairs have been used in the literature [24]. Elution with mobile phases containing strong carboxylic acids such as trifluoroacetic (TFA), pentafluoropropionic (PFPA), and heptafluorobutyric (HFBA) are common, improving both peak shape and sensitivity [24].

2.1.3 Ion generation

The eluting species from the liquid chromatography column can be ionised to form precursor ions by a variety of techniques. Liquid chromatography (LC) coupled with mass spectrometry is a common platform for development of analytical methods. The key essentials of an LC-MS system include the autosampler, the HPLC system, the ionisation source and the MS [25]. Several ionisation sources exist, interfacing between the LC system and the MS. Two of the most common are Electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI). Ionisation for both of these sources occurs at atmospheric pressure, therefore they are often referred to as atmospheric pressure ionisation (API) sources [25].

In bioanalysis, soft ionisation in the form of ESI is a common method used. It causes little fragmentation upon ionisation, allowing weak bonds to be preserved and the molecular ion to be observed. ESI works well with polar molecules therefore suited to tobramycin analysis.

ESI occurs in three defined steps; droplet formation, shrinkage of the droplet, and formation of the gaseous ions [38], as shown in Fig. 2.5. In the first step, the column effluent from the LC system is pumped through a needle and nebulised at the tip of a metal nozzle [39]. This high electric potential between the needle and nozzle forms a Taylor cone at the tip which is saturated with either positive or negative ions. A voltage between 3 to 5 kV is maintained at the tip which results in the analyte being dispersed from the Taylor cone as a fine spray of charged droplets by the electric field focused towards the MS. These droplets get smaller as they approach the entrance to the mass analyser, due to liquid evaporation [40], and are assisted by a nebulising gas, such as nitrogen, which shears around the eluted analyte to also give a higher flow rate. As the droplets get smaller, the distance between the surface charges also become smaller and smaller, with the surface charge density increasing, and the radius decreasing, resulting in the formation of highly-charged microdroplets [40]. Gaseous ions are formed when the electric field strength within the droplets reach the Rayleigh limit, the point where it is energetically and kinetically possible to undergo Coulomb fission. Individual ions in the gas phase then appear via desolvation and are accelerated out of the ion source, entering the vacuum interface leading to the MS.

Mobile phase composition can have a substantial impact on the level of ionisation, and in turn sensitivity of a method. Ion pairing additives such as formic acid (FA) can enhance ionisation efficiency of ESI. Organic modifiers are added to lower the polarity of the aqueous mobile phase, resulting in increased eluting strength in reversed phase chromatography [28]. Acetonitrile and methanol are the two most widely used organic modifiers as their mixtures with water have low viscosity reducing the column pressure as well as enhancing ESI [33]. Specifically, for tobramycin in the literature, ESI has been used frequently as Michele Xuemei Guo *et al. (2006)* [8] and Liusheng Huang *et al. (2018)* [20] report. ESI MS detection of ion pairs is not often ideal, and since ion pair chromatography is ideal for tobramycin, the sensitivity of MS will be reduced due to suppression of ionisation [19].

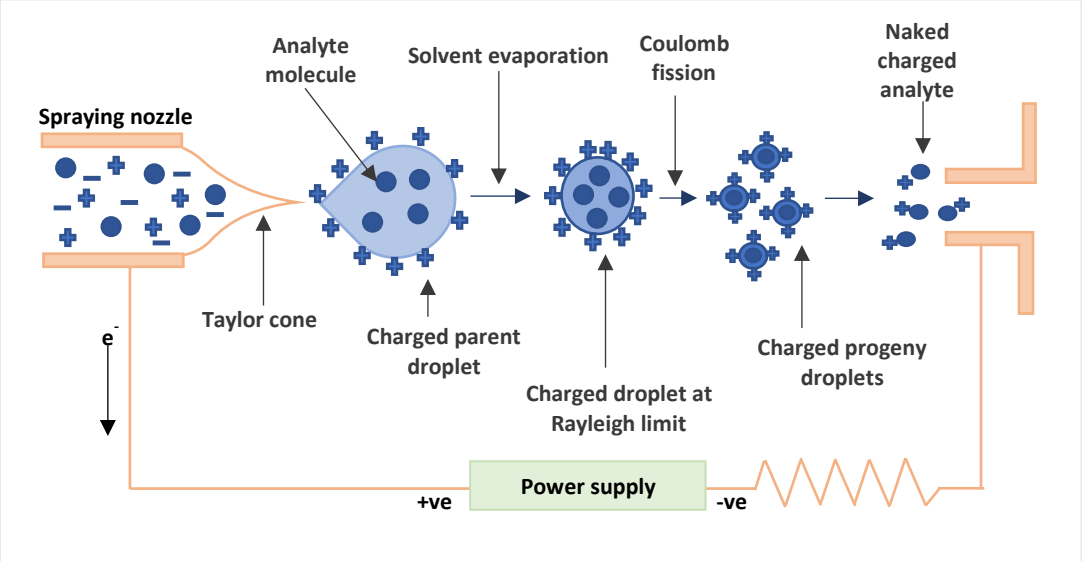


Figure 2.5: *Schematic illustrating the mechanism of an electrospray ionisation source. Image taken from http://lab-training.com/2016/01/21/popular-ionization-techniques-in-mass-spectroscopy/*

2.1.3.1 Mass Spectrometry & Tandem Mass Spectrometry

HPLC coupled with MS is one of the main analytical techniques of choice in drug discovery [25]. The main elements of an LC-MS system include the autosampler, the HPLC system, the ionisation source and the mass spectrometer [25]. Although there are several types of MS available for interfacing with HPLC systems, this report will focus on triple quadrupole MS-MS systems. These MS systems are most often used for quantitative bioanalytical systems [25], and thus are a suitable option for tobramycin quantification.

A MS is an instrument which separates gaseous ions according to their mass-to-charge (*m/z*) ratios, providing a result measuring the relative abundance of each ionic species present in the sample [41]. The analytical technique provides both qualitative and quantitative information [42]. The components of a MS consist of a sample inlet, an ionisation source, a mass analyser and a detector, within a vacuum. The vacuum prevents the ions generated from colliding with other forms of matter during the separation process, as this may alter the ion's direction of travel and ultimately stop it from reaching the detector [41]. The sample is first introduced into the ionisation source, to be ionised and

acquire positive or negative charges. The ions then travel through the mass analyser, which sorts the ions by their masses by applying electromagnetic fields [41]. According to their m/z ratio, they arrive at various parts of the detector making contact, which generates a signal recorded by a computer system [41]. This signal is graphically represented as a mass spectrum showing the relative abundance of the signals according to their m/z ratio [41].

Several different mass analysers are available; however, the principles of their process are similar. A triple quadrupole has three quadrupoles arranged in a linear series, and is one of the most common types of MS used for tandem MS/MS. A quadrupole consists of four parallel cylindrical rods, with each opposing pair connected via an oscillating electric field. An opposite but equal direct current (DC) or radio frequency (RF) potential is applied, resulting in an electric field that causes the ions to have oscillatory motion in the x-y plane whilst travelling forwards. Manipulation of the DC and RF voltages applied to the rods, controls the amplitude of an ion's oscillations dependent upon their m/z ratio. At given values of these potentials only ions of a certain *m/z* will have stable oscillation amplitudes and the ability to travel forwards through the quadrupole, eventually reaching the detector. Simultaneously, at these same potentials, other ions (not at the optimal m/z) will have large, unstable oscillation amplitudes, resulting in contact with the rods. They will be neutralised and not reach the detector.

Specifically, in triple quadrupole mass analysers, each cell plays a role to increase selectivity of ions and denoted as MS/MS (Fig. 2.6). Ionised samples introduced via the sample inlet, enter the first quadrupole (Q1) where the analyte ion of interest (parent/precursor) is mass selected [42]. In a triple quadrupole MS only Q1 and third quadrupole (Q3) have this capability to select based on *m/z*. The precursor ion selected is then allowed to collide with a collision gas in Q2 (e.g. argon or N_2), often referred to as the collision chamber. The collision cell has as a fixed radiofrequency voltage and is the cell where collision-induced dissociation (CID) occurs [42]. The precursor ions are activated by collision and undergo further fragmentation, forming product ions with structural information (specific m/z ratios), which are monitored in Q3 [42] and by the detector.

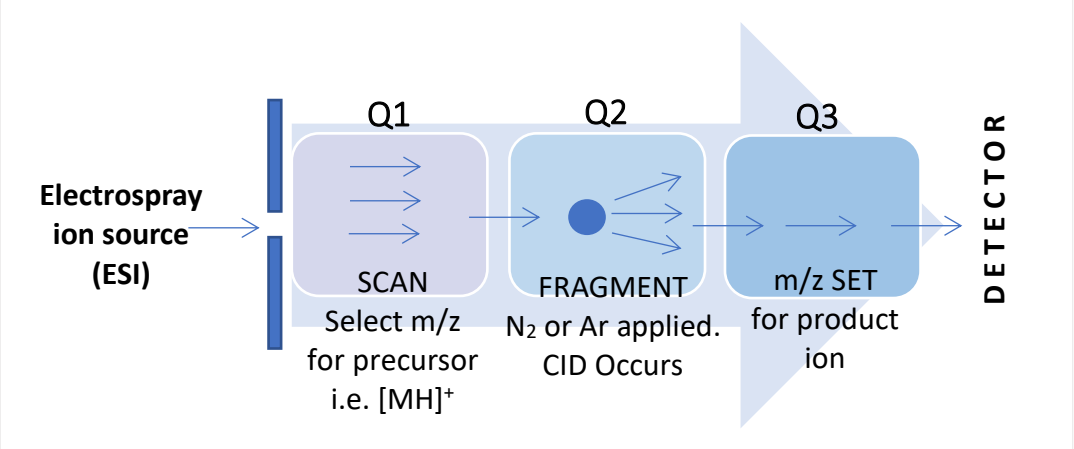


Figure 2.6: *Schematic illustrating a triple quadrupole mass spectrometer (MS/MS)*

2.1.3.2 Internal standards (IS)

Ion suppression as well as enhancement of ionisation can have an impact on the MS signal, as often more than one component, from the sample, buffers or mobile phases, can elute at the same time as the analyte of interest. Compromised MS signal via competition of ionisation process and variability in ion suppression can limit the ultimate sensitivity of an assay [39]. Biological samples are highly complex, containing fatty acids and lipids which have a high surface activity, therefore these components can be part of the cause of ion suppression effects. The effects result from alteration in droplet formation and evaporation efficiency due to the presence of less volatile compounds. The high surface-active matrices will out-compete the low surface-active analyte, where the outcome is an uneven fission process, affecting the number of ions transitioning to the gaseous phase. This reduces their ability in reaching the detector and therefore the analytes can become undetectable.

During quantitation, to control for this, as well as for extraction and autosampler variabilities, an internal standard is injected onto the system with the analyte. An internal standard has a similar structure and properties to the analyte of interest, either a structural analogue or a stable isotope labelled (SIL) IS [41], depending upon availability. A SIL is the gold standard as it is the compound of interest with substitutions of a few elements. A stable isotope which co-elutes with the analyte of interest is most favourable, as it will ideally have the same behaviour as the analyte of interest. When samples are being analysed a certain volume of internal standard is added to each one, this produces two

chromatograms for each sample, one for the analyte and one for the internal standard. The area under these peaks is calculated and an analyte peak area/internal standard peak area ratio is obtained. This ratio can be used for accurate quantitation as it compensates for any change in instrument sensitivity or error in injection volume throughout the analysis of the samples.

There are many different options for internal standards for tobramycin methods. Attema-de Jonge *et al*. (2007)[19] utilised sisomicin as an IS, due to its structural and physiochemical similarity to tobramycin predicting that both compounds should behaving in a similar manner during sample processing [8, 19]. Tobramycin can be separated into the kanamycin family, due to the structure presenting two amino sugars attached to the central 2-deoxystreptamine moiety; one of these being a 3aminohexose [24]. This family also includes kanamycin and amikacin, being viable internal standards with a similar structure to Tobramycin.

2.1.4 Sample Preparation & Extraction

Biological samples, for example serum/plasma samples from a clinical study, are complex matrices. Prior to injection onto the mass spectrometer, biological samples must undergo a level of clean up and extraction. This prevents the instrument from contamination and operational error whilst removing interference from high concentration proteins. Preferably sample extraction methods isolate and enrich the analyte of interest, removing as many interferents as possible without breakdown of any of the target compound chemical components or loss of the target compound through evaporation or adsorption. Due to the possibility of compound-protein and metabolite-protein interactions, the sample preparation method needs to be rigid and strong enough to remove these proteins, but gentle enough to leave the target compound intact [43].

The development of new LC-MS/MS methods for small molecules in biological fluids is routinely becoming more challenging, due to the continuous need to achieve higher sensitivity and assay robustness, alongside the added complexity of analysis of biofluids such as serum, plasma, urine and cerebrospinal fluid (CSF) [44]. Often there are low levels of pharmaceutical targets, resulting in the need to concentrate samples prior to analysis however, this also has a knock on effect of increasing the concentrate of interfering

components within the sample extract [44]. There are a few main factors encountered during analysis of small molecules from biological fluids which can hinder quantification via LC-MS/MS, two being matrix effects and phospholipids.

2.1.4.1 Matrix Effects

Matrix effects can cause an ion enhancement or ion suppression of the analyte response caused by co-eluting endogenous matrix constituents (e.g. metabolites of the target analyte, proteins or lipids), or exogenous compounds introduced during sample processing and analysis [44, 45]. For bioanalytical analysis where biological matrix, such as plasma, urine, blood, and tissues are analysed, matrix effects can have an impact upon precision and accuracy of a method [45]. Matrix effects can be determined by selectivity experiments comparing different batches of biological matrix, as well as post-column infusion of the analyte [44]. The presence of substances from the matrix interfering with analysis is calculated by comparing signals of a standard to a spiked matrix blank at the same concentration [46]. A variation in signal intensity at or near the retention time of the analyte, would indicate the presence of matrix effects interfering with the analyte signal [44].

2.1.4.2 Phospholipids (PL)

Phospholipids are involved in many biological transport processes and are the main constituents of cell membranes, and so present in many biological fluids such as plasma and serum [46]. PL are the main source of matrix effects in LC-MS/MS bioanalysis causing ion suppression. This is linked to competition for space on the surface of droplets formed during the ESI process [44], as explained in section 2.1.3. As PLs can be present in different concentrations, and can impact assay robustness, screening for their presence is important during method development.

Effective sample preparation requires knowledge of the target analyte structure and physical and/or chemical properties, as each individual technique exploits the properties of the target analyte to facilitate separation from its surrounding matrix. Properties that are used most widely explored are ionisation state, pK_a, solubility, LogP, hydrophobic and hydrophilic interactions and ionic interactions. For Tobramycin, its physical properties are explained in section 1.1.5, which indicate that it is extremely hydrophilic and possesses a

high number of amino and hydroxyl moieties in the carbon chain. A basic substance with a pK_a of approximately 6.7-9.9, tobramycin will be stable at high pH or very low.

A variety of sample extraction methods for plasma have been used with LC-MS/MS for additional selectivity and clean up. These include protein precipitation (PPT), liquid-liquid extraction (LLE), solid phase extraction (SPE) and immunoaffinity purification [25].

2.1.4.3 Protein Precipitation (PPT)

One of the common processes used in drug discovery is de-proteinisation by protein precipitation, which aims to reduce matrix effects, and help improve sensitivity by concentrating the analyte of interest. The advantages to PPT are time, cost as it's relatively cheap, and the option of automation [25] as well as, suitability for both lipophilic and hydrophilic analytes [47]. PPT extracts the plasma with a common organic solvent, e.g. MeOH and ACN, to denature the plasma proteins of high abundance and high molecular weight. The organic solvent, added in a certain ratio [43], reduces the water solvation of some of the proteins, resulting in their hydration, aggregation and formation of a solid precipitate. Following centrifugation, the analyte of interest remains in the supernatant whilst the proteins present are concentrated in the pellet can be separated from the liquid phase.

PPT coupled to LC-MS/MS provides a simple, rapid and inexpensive technique [47], for high throughput analysis, however unfortunately the process can be nonselective, with endogenous proteins still being present post sample clean-up. This can lead to ion suppression, matrix effects and endogenous peaks interfering with MS/MS detection [47].

As AGs are not extensively bound to any specific plasma proteins, PPT is a viable option for a non-labour intensive, fast, sample clean-up method. PPT recovery data for tobramycin using Acetonitrile as an organic solvent have previously been published.

2.1.4.4 Solid Phase Extraction (SPE)

Solid Phase Extraction (SPE) is a rapid preparation technique that manipulates the ionisation state, hydrophilic and hydrophobic interactions and *pKa* of a molecule to separate it from the rest of the matrix. SPE is a costly, multiple step method consisting of sorbent conditioning, adsorption or loading, washing to remove non-specific binding

molecules and then elution of the target molecule from the phase [43]. The technique works via portioning between the solid and mobile phase via SPE cartridges, like conventional liquid chromatography. SPE sorbents fall into four distinct categories, non-polar, polar, ion exchange (cation and anion), and mixed mode.

Non-Polar SPE sorbent works with non-polar functional groups such as C_{18}, C_8, and phenyl, and are used for the extraction of molecules which contain non-polar functional groups via Van der Waals forces between the analyte and sorbent surface. Polar solvents facilitate extraction repelling the analyte from the solution phase and more strongly onto the sorbent surface. Elution occurs via disruption of interactions between the analyte and SPE functional groups achieved using non-polar solvents e.g. MeOH and ACN. Polar SPE extracts polar analytes from non-polar matrices through interactions with polar sorbents on the surface of the cartridge e.g. aminopropyl, cyanopropyl and unbonded silica. Dipole-dipole or hydrogen bonding interactions allow retention of analytes with polar functional groups.

The availability of cartridges with large particle size sorbent allows for rapid sample preparation. Easily automated, and very popular in industry, SPE overcomes the limitations of PPT, working to remove contaminations and enrich the sample [48].

2.2 Development of a LC-MS/MS Method for Tobramycin

2.2.1 Method Development Aims

As explained, there is a need for a rapid and simple method for tobramycin quantification in biological samples. Currently, the bioanalytical methods available are timely, and require specialised equipment, however delivery of a simple LC-MS/MS assay with a comparable LLQ will aid future tobramycin quantification and monitoring in pre-clinical and clinical samples.

This work will focus on devising a sensitive and reproducible bioanalytical method for the quantification of Tobramycin. The aim is to develop a LC-MS/MS methodology for determining Tobramycin concentrations in biological matrices. Rat plasma will be utilised to demonstrate the capacity of the LC-MS/MS method.

2.2.2 Objectives

A LC-MS/MS assay will be developed for a tool compound Tobramycin. This will involve:

- Tuning the mass spectrometer to select specific transitions for tobramycin (tool compound) and kanamycin (chosen internal standard)
- Optimising the LC system, investigation column and mobile phase
- Investigating sample preparation methods of protein precipitation for suitable extraction of tobramycin from rat plasma
- Developing an appropriate calibration range and QCs

2.3 Materials & Methods

2.3.1 Materials

2.3.1.1 Reference compounds, chemicals and biological specimens

Tobramycin (CAS No 32986-56-4) and Kanamycin (CAS No 70560-51-9) were used at 1 mg/mL and purchased from Sigma-Aldrich. In addition, trifluoroacetic acid (TFA, LC-MS ultra-grade), dimethyl formamide (DMF, 99.8 %), ACN and MeOH (both at HPLC grade) were also purchased from Sigma-Aldrich. Heptafluorobutyric acid (>95 %) was purchased from Thermo Scientific. Ultrapure water (18.2 MΩ cm) was prepared with an ELGA Purelab Ultra water purification system (ELGA LabWater). A Jasco PU-960 Intelligent HPLC pump was used for direct infusion of tobramycin into the MS. A drug free biological matrix, Han Wister rat plasma (K2 EDTA), was utilised as a model matrix within this analysis, purchased from Marshall Bioresources and stored at -20 °C.

2.3.1.2 Instrumentation

LC separation was performed with a Waters Acquity™ HPLC system (Waters Corporation) and consisted of a fixed 10 µL loop autosampler, binary solvent manager, sample organiser and column manager. The chromatographic gradient was utilised and designed to elute analyte of interest around the midpoint of the total run time. The autosampler temperature was maintained at 4 °C and operated in partial loop mode.

Detection was undertaken using a Mass Spectrometer Applied Biosystems Sciex API-5500™ triple quadrupole MS (Sciex, Warrinton, UK), operated in positive mode. Acquisition and inlet methods differed per experiment. MS scan type was multiple reaction monitoring (MRM). Analyst Version 1.6.1 (Applied Biosystems/MDS Sciex, Canada) was used for data acquisition and processing. The columns utilised were Phenomenex Synergi™, 50x3 mm, 4u Polar, and Hydro-RP Mercury, 2x20 mm, 2.5 um, and an Acquity™ UPLC® CSH C18.

2.3.2 Methods

2.3.2.1 Standard Preparation

The quantification of analyte concentrations in study samples is achieved through comparison of sample MS responses against those recorded from a calibration line,

prepared from standards of known concentrations. Quality control (QC) points are used to ensure accuracy and independently validate the standard calibration line per run. Each analytical run is subject to precision and accuracy (P&A) acceptance criteria. For the method documented in this report, the acceptance criteria adopted in a discovery environment was implemented, of +/- 20% from the nominal concentration, validating comparisons between assay runs.

Each experimental run requires the preparation of a calibration line, through serial dilution of a stock solution, spanning the dynamic range for the assay. The bioanalytical procedure is as follows,

1. Preparation of independent A and B Tobramycin stock solutions in DMF from a single solid Tobramycin stock. Independent stocks allow accuracy of weighing to be determined, as one with be used for calibrants and one for QCs.
2. Infusion onto the MS to allow for specific optimisation to build a robust assay to support bioanalysis.
3. Sample preparation: preparation of a standard calibration line (using stock A) and QCs (using stock B) in respective matrix, as per the study protocol.
4. Extraction of analyte from study samples, standards and QCs via PPT.
5. Injection, detection and quantification of study samples, standards and QCs via HPLC-MS/MS technology.

Manual preparation of standards and QCs typically utilises the method of serial dilution of compound stock solutions (in Acetonitrile (ACN):H_2O (1:1 v/v)) to prepare working solutions (Table 1), which are subsequently spiked into biomatrices to achieve desired analyte concentrations (Table 2). A 50 µl sub aliquot of each standard is taken into fresh tubes and an IS in an organic precipitation reagent is added. These are then centrifuged for approximately fifteen minutes to produce supernatant, which is transferred into fresh tubes for analysis on LC-MS/MS.

The IS stock solution for kanamycin was prepared at 5 mg/mL by dissolving the required amount in DMF. The IS working solution for sample extraction was prepared at 1 µg/mL by diluting 40 µL of stock solution with 40 mL of 0.2% TFA in Methanol. The stock solutions for both tobramycin and kanamycin were stored at 4 °C.

Table 2.2: *Example table of working solution preparation by serial dilution of stock solutions.*

Working Solution	**Final Concentration (µg/ml)**	**Volume of Spiking Solution (µl)**	**Volume of 50:50 ACN:H_2O (µl)**
A/B	1000		
A1/B1	100	50 of solution A/B	450
A2/B2	10	50 of solution A1/B1	450
A3/B3	1	50 of solution A2/B2	450

Note: Working solutions: A for Standards, B for QCs

Table 2.3: *Example spiking scheme using working solutions made in Table 3.1 to create calibration line.*

Standard Concentration (ng/ml)	**Volume of Working Solution (µl)**				**Volume of control matrix (µl)**
	A3	A2	A1	A	
	1 µg/ml	10 µg/ml	100 µg/ml	1000 µg/ml	
10	5				495
50	25				475
100		5			495
500		25			475
1000			5		495
5000			25		475
8000				4	496
10000				5	495

Example of spiking scheme for creating standard calibration lines in control matrix via serial dilution of single stock solution to prepare working solutions. Single stock solution A was prepared to 1 mg/ml in DMF and serially diluted 10-fold (in (1:1 v/v) ACN: H_2O) to produce working solutions (A1-A3). Respective volume of working solution was spiked into control matrix to achieve a 500 µL bulk of desired nominal standard concentration as per the spiking scheme.

The spiking scheme ensures that the percentage of organic solvent in each aliquot does not exceed 5% of the total assay volume. This is important as calibration standards and QCs should be prepared to mimic biological study samples as closely as possible. In this regard, the organic solvent content should be minimised, containing no more than 5% solvent and mimic any additives that study samples may also contain.

2.3.2.2 Extraction Techniques

Protein precipitation

An identical volume of 200 µL IS solution was added to each 25 µL calibration standard, washes and blanks for all preparation techniques via a multi-shot electronic pipette. An identical volume of ACN was added to double blanks. All tubes were capped and vortexed for approximately 3 minutes. Samples were then centrifuged for a minimum of 10 minutes at 3000xg, until there was clear separation between supernatant and precipitate. 100 µL of supernatant was then manually transferred to new 1ml Micronics tubes for injection onto the HPLC-MS/MS system.

2.4 Fragmentation & Infusion

2.4.1 Fragmentation Pattern

The chemical structure of tobramycin and its proposed ESI-MS/MS fragmentation pathway is shown below in Fig. 2.7 [49] [50].

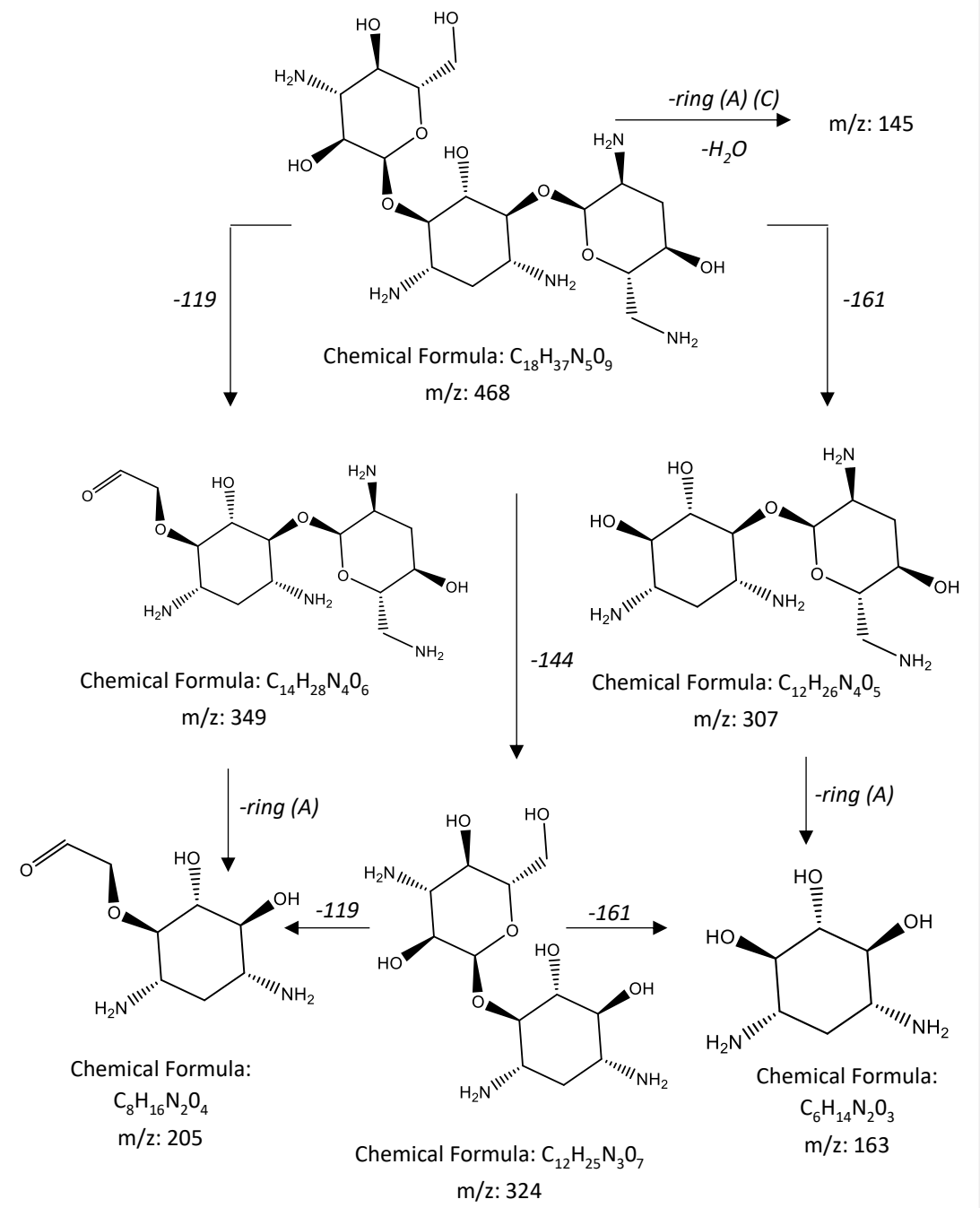


Figure 2.7: *Proposed fragmentation pathway of tobramycin.*

Image adapted from [49] [50].

Tobramycin showed three major fragmentation pathways, based on rings A, B and C. Cleavage between rings A and B of tobramycin resulted in the most abundant product ion at m/z 324, by the loss of 144 Da [49] [50]. This ion further fragmented by the cross-ring cleavage involving ring C and the cleavage between rings B and C, to produce product ions at m/z 205 and 163 by the respective loss of 119 and 161 Da. The less abundant product ion at m/z 307 by the loss of ring C (-161 Da), also yielded the product ions at m/z 163. The product ion m/z 349, formed by the cross-ring cleavage of ring C, was also detected [49] [50].

2.4.2 Direct Infusion

Quantitative HPLC-MS/MS is carried out by detecting analytes according to their parent to product ion transitions, known as MRM. Tobramycin was infused at 20 ng/mL (in (1:1 v/v) ACN: H_2O) directly on an API 5500 MS with an external pump. The infusion pump was used at a flow rate of 0.8 mL / min into the turbo ESI ion spray ion source of the MS. To determine the MRM transitions, firstly a Q1 scan was performed, providing m/z ratios for the precursor ions across the range of 320 to 500. Tobramycin yielded a $[M+H]^+$ base peak at m/z 468 [50], calculated from its molecular weight (mw) of 467.52, at an intensity just under 2.0e6 (Fig. 2.8). The mass spectrum shows significant peaks, resolved as isotope clusters well separated from one another.

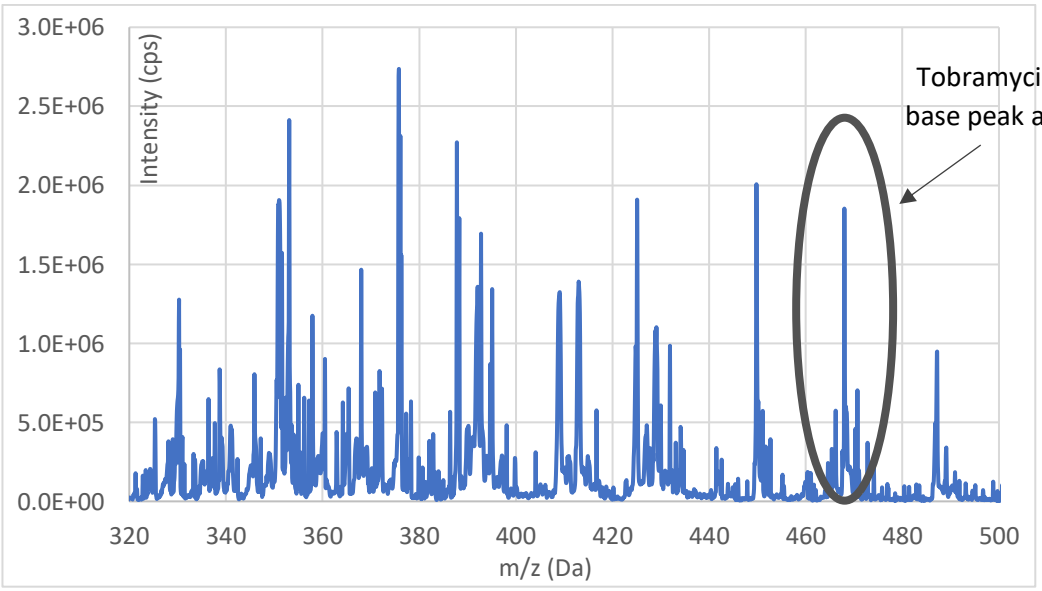


Figure 2.8: *Tobramycin full Q1 scan across the mass ranges of 350 – 500, infused at 20 ng/mL (in (1:1 v/v) ACN: H2O) at a flow rate of 0.8 mL/min.*

Once the parent ions were determined, they were fragmented through collision with nitrogen gas in the collision cell of the MS to determine their product ions. The precursor ion (m/z 468.3) will break up into fragment ions of a lesser molecular weight when a collision energy is applied during a product ion scan. In Fig. 2.7. the proposed fragmentation pattern for tobramycin is illustrated, and on direct infusion these abundant product ions were seen at m/z ratios of 324.4, 163.1 and 205.2. These product ions are a result of cleavage of the glycosidic bonds and the subsequent loss of the amino sugar rings [8, 19] (Fig. 2.9).

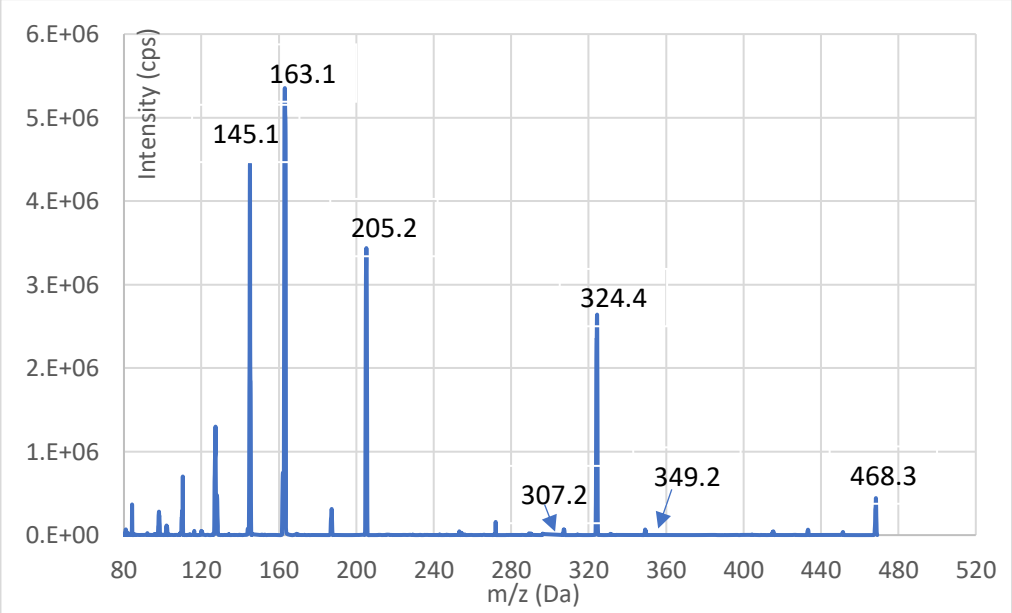


Figure 2.9: *Product ion scan (MS/MS spectra) of m/z 468.3, at a collision energy (CE) of 25.*

The most abundant product ion observed was 163.1, resulting in the MRM transition of 468.3/163.1 for tobramycin. This MRM transition identified was also utilised by Milly E. Attema-de Jonge *et. al*. [19] and Dan Shou *et al.* [23], as well as cited in many other methods in the literature.

Alongside determining the parent to product ion transition, specific MS parameters were optimised to ensure the highest intensity of signal was obtained. The optimised parameters utilised, are given in Table2.4.

Table 2.4: *MS Parameters*

Parameter	Effect	Result
Curtain Gas (CUR)	The CUR is a stream of heated inert gas, that aids in the evaporation of solvent from the sprayed particles and to maintain the vacuum.	35.00 psi
Gas 1 (GS1)	GS1 is the nebulizer gas. It helps to generate small droplets that rapidly desolvate in the ion source.	30.00 psi
Gas 2 (GS2)	GS2 blows out of the ceramic heaters and helps to evaporate the spray droplets.	50.00 psi
Delustering potential (DP)	The orifice is the opening where ions enter the mass spectrometer. The DP is a voltage applied to the orifice that helps to prevent the ions from clustering together.	120 V
Ionspray voltage (IS)	The IS is applied to the tip of the ionspray needle, ionising the sample.	5500.00 V
Temperature (TEM)	TEM is added through the ceramic heaters to help to rapidly evaporate the spray droplets.	550°C
Entrance Potential (EP)	The EP is applied to the first set of quadrupoles past the orifice plate. These rods do not act as mass filters but serve to guide and focus the ions into the MS.	10.00 V

Collision Energy (CE)

CE refers to the rate of acceleration as the ions enter quadrupole 2 (Q2). The ions undergo a thermal interaction with the collision gas and fragment, as explained in Fig. 2.6. The higher the CE, the greater the level of fragmentation and the potential for product ions. If the CE is too low, the molecule undergoes inefficient fragmentation, however raising the CE too high can cause too much fragmentation. The CE is a central parameter that can impact the sensitivity of an MRM method.

Fig. 2.9. was acquired with a CE of 25. To ensure optimal CE was obtained for the method and the precursor ion (m/z 468.3) was completely fragmented in the collision cell, a CE of 20 and 30 were tested (Fig. 2.10 and 2.11).

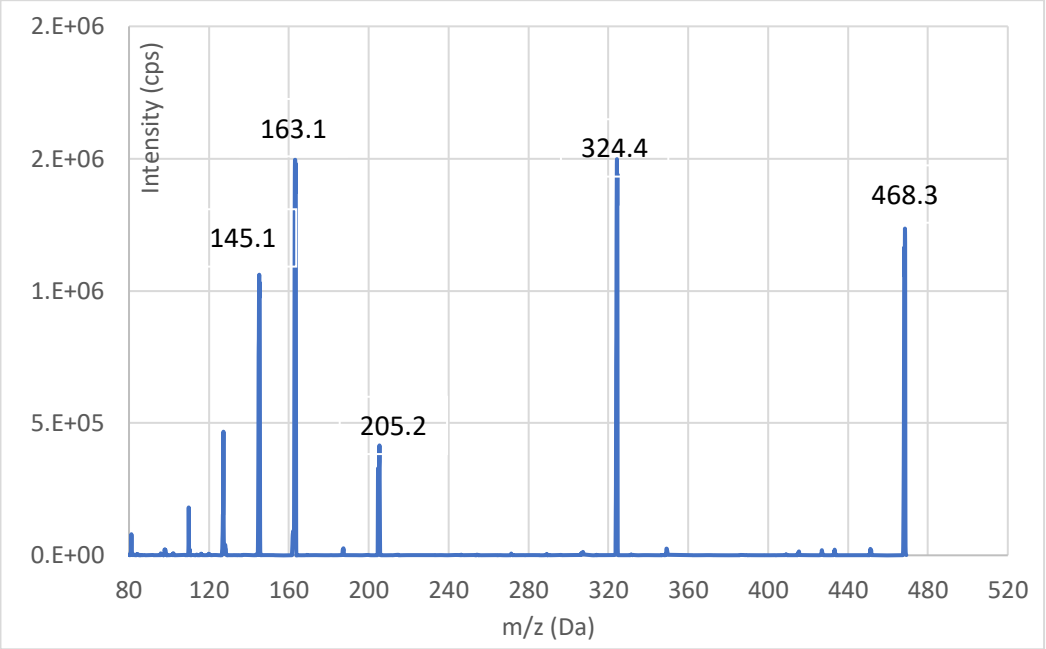


Figure 2.10: *Product ion scan (MS/MS spectra) of m/z 468.3, at a collision energy (CE) of 20.*

At a CE of 20, inefficient fragmentation has occurred with the presence of approximately 1e6 of precursor ion. In comparison to Fig. 2.9, the intensity of the selected product ion m/z 163.1 has decreased. This signifies that CE 20 is not the optimal CE for this method.

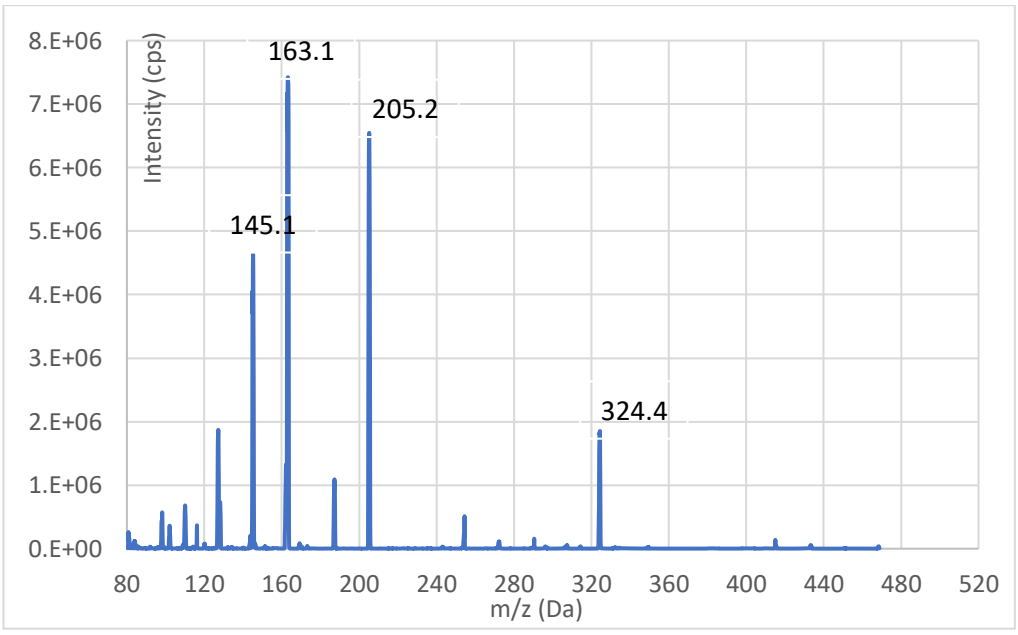
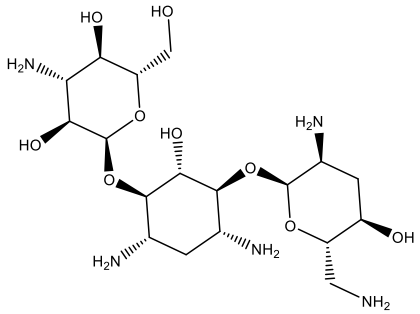
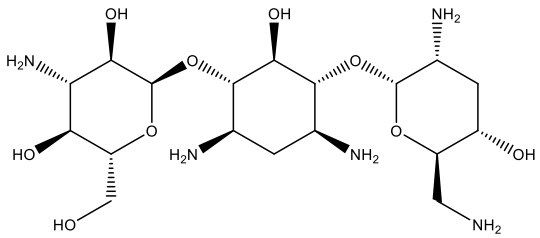


Figure 2.11: *Product ion scan (MS/MS spectra) of m/z 468.3, at a collision energy (CE) of 30.*

At a CE of 30, the precursor ion is no longer distinguishable on the spectra. This highlights that complete fragmentation has occurred and implies that a CE of 30 will be fit for purpose for this method.

The table below defines the final mass transitions selected for both tobramycin and kanamycin, the chosen IS for this method (Table 2.5). As explained previously, Kanamycin is within the same family of compounds as tobramycin, making it a viable IS choice from its structure being similar without the introduction of any new functional groups. Kanamycin was infused in the same manner as tobramycin.

Table 2.5: *Test Compounds used and their MS/MS transitions.*

Compound	MS/MS MRM Transition (Positive Ion Mode)	Structure
Tobramycin	468.3/163.1	
Kanamycin (Internal Standard)	485.5/163.1	

2.5 Results and Discussion

2.5.1 Detecting Tobramycin

To produce a LC-MS/MS method for quantification of tobramycin, selecting a stationary phase producing a clean MRM chromatogram was important. This entailed a robust method producing a low background signal, clear separation, high resolution and sensitivity. Reverse phase columns have been reported to be suitable for compounds with a variety of physiochemical properties. The column was evaluated based on background, peak shape and ability to retain tobramycin. Factors of analyte separation, retention, resolution and the mobile phases used were considered.

Following an existing LC-method (Table 2.6 and 2.7) developed in 2017 by a colleague in the US, an initial experiment was conducted to determine reproducibility of the method in the UK. This is important because reproducibility reflects the precision and robustness of a method between different sites, determining successful method transfer.

Table 2.6: *Existing LC Method Conditions developed in 2017*

Mobile Phase A	**0.1% HFBA in water**
Mobile Phase B	0.1% HFBA in Acetonitrile
Sample Temp (°C)	4
Column Temp (°C)	RT
Flow Rate (mL/min)	0.8
Injection Volume (µL)	4

Table 2.7: *Existing Mobile Phase Gradient (Inlet Method) developed in 2017. The total run time was 2 minutes at a flow rate of 0.8 mL/min.*

Time (min)	%A	%B
Initial	90	10
0.1	90	10
0.6	10	90
0.9	10	90
0.95	90	10
1.4	90	10

The extraction method used tobramycin preparations at 1 mg/mL which were dissolved in DMF and working solutions were prepared as detailed in Table 1 in Methods section. A blank (50/50 ACN: H_2O) and one calibrant was prepared at 2000 ng/mL in 50/50 ACN: H_2O following the method described in Section 2.3.2. This concentration was chosen as existing methods presented an analyte peak of high signal/background, and thus worked as starting point for ensuring the method was reproducible for tobramycin. Sample preparation following PPT was conducted and 5 µL was injected onto the HPLC.

The existing method utilised a Synergi™ Hydro-RP Mercury (2x20 mm, 2.5 µm) column, with the addition of HFBA to the mobile phase and detection via a Sciex™ API 4000 Q TRAP MS, all of which were not available here in the UK. The benefits of a Q trap instrument allow for the Q3 to be used as a quadrupole or an ion trap, enhancing selectivity and resolution at reduced scan speeds. Therefore, within this first experiment the Initial chromatographic analysis of tobramycin was performed on a Phenomenex, Synergi™ 4 µm Polar-RP 80 Å, LC Column 50 x 3 mm. This column was chosen as it is recommended for separation of polar compounds, which is the case with tobramycin. Due to the unavailability of HFBA at the time, a mobile phase composition of 0.1% FA in H_2O: ACN was utilised, which are common buffers used in generic method development. For detection a Sciex™ API 5500 MS was used which can scan in a mass range of m/z 5 – 1250 in quadrupole mode. A typical chromatographic response for a blank sample and tobramycin is shown in Fig. 2.12.

2.5.2 Chromatographic Detection

As can be seen in Fig. 2.12 a blank sample prepared in 50/50 ACN/H_2O showed background noise, over multiple injections. This could be due to the mobile phases used, the flow rate, the column or the selectivity of our MRM. Ultimately, this has the potential to obscure sample peaks, decrease sensitivity of our assay and raise a future LLOQ.

The 2000 ng/mL tobramycin calibrant prepared in 50/50 ACN/H_2O, showed a peak at ~0.75 mins and was well retained on the column (retention factor of 2), before eluting and resolved from any other peaks in the sample. However peak tailing is noticeable from the chromatogram, as can be seen in Fig. 2.12. as well as the peak shape not being the ideal, symmetrical shape of a Gaussian peak.

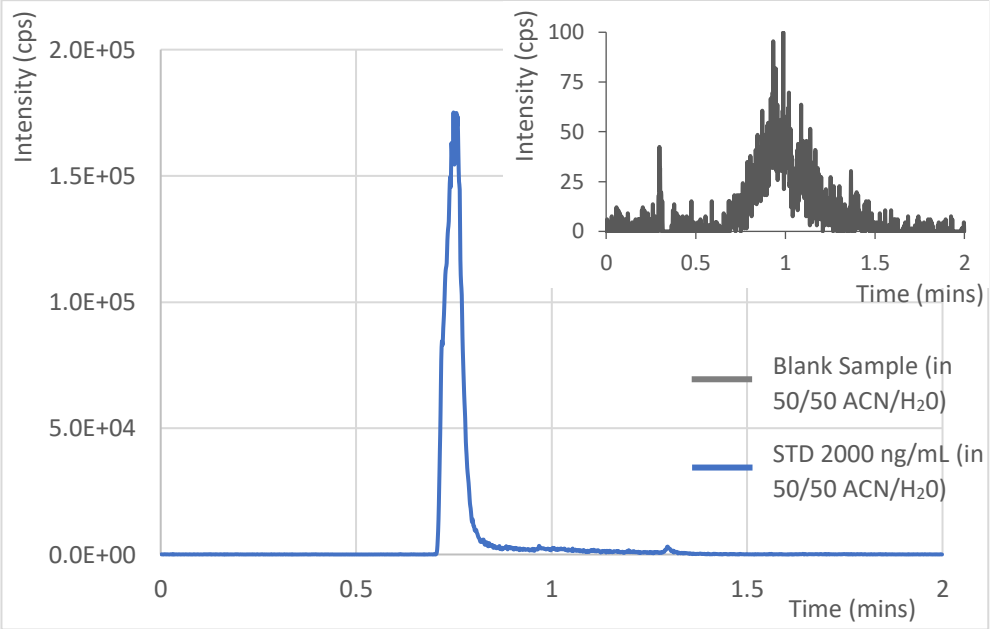


Figure 2.12: *Typical chromatographic response of a blank sample and a standard at 2000 ng/mL tobramycin in 50/50 ACN: H_2O, On a Phenomenex, Synergi™ 4 µm Polar-RP 50 x 3 mm column, at a flow rate of 0.8 mL/min. A peak is noted at ~0.75 mins for tobramycin.*

Peak Tailing

Peak tailing is a common peak shape problem since the early days of RP-HPLC. Almost every chromatographic peak can tail to a certain degree, however since it can affect the quality of the separation, quantifying tailing can aid in method development [51]. A gaussian peak has a tailing factor (T_f) equal to one, as it possesses absolute symmetry. Generally, when asymmetry factors are greater than 2.0, then peak tailing must be addressed. Peaks tail because of overloading of retention mechanisms, and interaction with acidic or ionised silanol groups on the surface of the silica particles within the column [51]. Fig. 2.13 highlights peak asymmetry and peak tailing calculations.

The tobramycin peak in Fig. 2.12 has a peak tail of $T_F < 1.2$. Tailing of this magnitude is known to have negligible effect on separation. Within this case, the minor peak tail is most likely due to insufficient mobile phase additive or the wrong mobile phase pH.

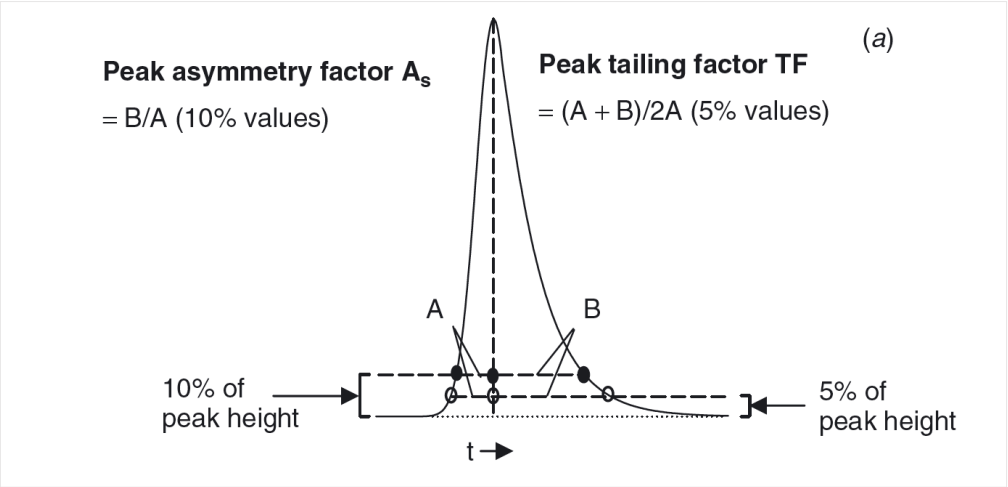


Figure 2.13: *Schematic illustrating peak asymmetry and tailing factors (A_S and T_F).*

Image taken from [52].

In their research, Mokh, S. et al, noted that aminoglycoside quantification was mainly hindered by poor rentention and peak shape, and improved with the addition of a volatile ion-pair reagent (e.g. TFA, PFPA or HFBA) in the mobile phase composition, aiming to regulate the retention of polar compounds on the stationary phase [21]. Taking this into consideration, optimisation of the mobile phase composition was carried out to improve the resolution of the peaks. The aim was to identify mobile phases where the compounds were retained on the stationary phase, yet elution was not unnecessarily extended.

2.5.3 Heptafluorobutyric acid (HFBA) addition

In RP-chromatography, ionic compounds are usually not retained by hydrophobic stationary phases. By adding an ion-pair reagent with an ionic end and a hydrophobic tail to the mobile phase, the hydrophobic tail of the reagent gets retained by the stationary phase, forming an ion exchange group on the surface of the stationary phase. The ion pairing agent must be oppositely charged than the analyte and must have good hydrophobicity to enhance peak shape and retention. HFBA is a strong carboxylic acid with anionic ion-pairing capability. The heptafluorobutyrate (HFBA−) anion will interact and neutralise positively charged peptide residues in the tobramycin structure (arising from the basic side-chains Lys, Arg and His, or a free α-amino group) [53]. This will decrease peptide hydrophilicity, and in turn increase the affinity of the peptides for the reversed-phase sorbent [53].

To address the peak tailing observed, and decrease the variables from the existing method, HFBA was sourced and added to the mobile phase. Addition of ion-pairing reagent HFBA to the mobile phase, should improve the chromatographic response by stabilising retention and minimising peak tailing due to ionic interactions. Therefore, chromatographic analysis of a 2000 ng/mL tobramycin calibrant prepared in 50/50 ACN/H_2O was investigated utilising HFBA within the mobile phase. Chromatographic analysis of tobramycin was performed using the same parameters as outlined previously in Table 2.7, however the mobile phase utilised was, mobile phase A 0.1% HFBA in H_2O, and mobile phase B was 0.1% HFBA in ACN.

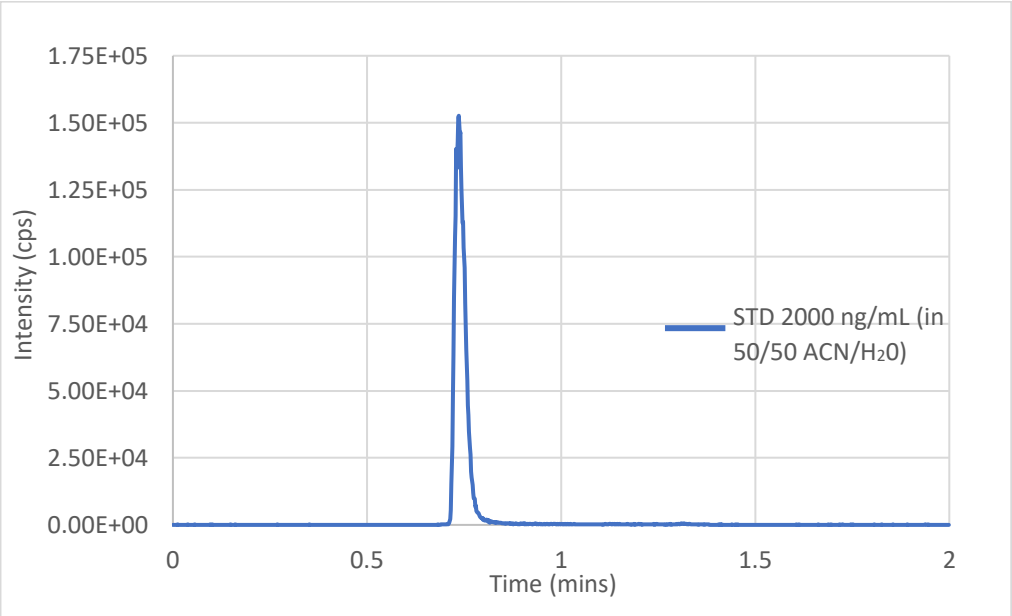


Figure 2.14: *Separation of Tobramycin on a Phenomenex, Synergi, 50x3 mm, 4u Polar column with the addition of HFBA in mobile phase buffers.*

The addition of an ionic surfactant to RP-chromatography can affect retention and selectivity of ionic compounds. In this case, the addition of an ion-pairing reagent, HFBA, to the mobile phase, has improved both the peak shape and the tailing noted in Fig. 2.12.

J.W. Dolan (2008)[54] states that some precautions must be taken when using ion pairing reagents. Recommendations for dedication of a single column for ion-pair reagent work is common, as Ion-pair reagents can never be fully washed off from a column [54]. Trace elements remaining can alter sensitivity of other methods where an ion pair has not been used [54]. It is also important to allow enough time for the mobile phase to equilibrate with the column, avoiding temperature changes disrupting the equilibrium [54].

As the addition of HFBA improved both the peak shape and retention of tobramycin prepared in 50/50 ACN/H_2O, the next step was to introduce matrix. For this method, rat plasma was utilised as a model biological matrix, as future *in vivo* samples would be in this format. For this a blank (rat plasma) and one calibrant was prepared at 2000 ng/mL in rat plasma following the method described in Section 2.3.2. The blank sample was intended to assess the signal/background with rat plasma.

Signal/background (S/N)

Signal-to-noise (S/N) ratio, is a measure of sensitivity of a method and often used to determine the limit of quantification and system suitability in LC-MS/MS. S/N can have an influence upon the performance of a method. S/N can be calculated in many ways but is typically defined as the signal being the height of the peak and the noise is the breadth of the baseline. The signal is measured from the midpoint of the noise to the top of the peak. S/N is obtained by dividing the signal by the noise. The S/N ratio can be used as an indication of whether an analyte is above the limit of detection (LOD) of a standard preparation, producing a signal peak larger than the noise. If the S/N ratio is equal to three then it is said to be above the detection limit, proving the presence of the analyte in the test sample with a probability larger than 99%. The quantification limit corresponds to a standard concentration which results in S/N ratio equal to or greater than five in our bioanalytical labs. In Fig. 2.14 the tobramycin signal in the calibrant prepared is great than five times higher than the blank.

2.5.4 Matrix addition

Tobramycin was injected at 2000 ng/mL in rat plasma, following the method outlined in Table 2.7. Sample preparation following PPT was conducted and 5 µL was injected onto the HPLC. At this concentration, it was not possible to determine the peak of interest as the addition of rat plasma had an impact on the chromatographic peak. To deduce a retention time for tobramycin, a STD of higher concentration was injected (Fig. 2.15).

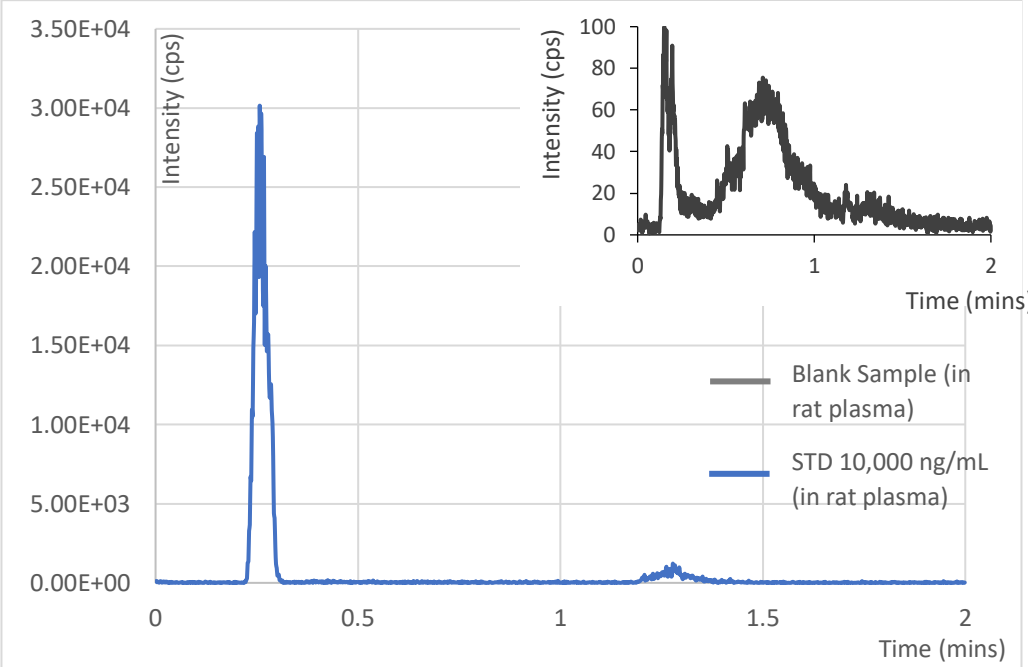


Figure 2.15: *Separation of Tobramycin following the addition of rat plasma.*

Following the first few injections poor retention was noted, and tobramycin was eluting early, near the solvent front, at an approximate retention time of 0.26 mins. There is an apparent difference between Fig. 2.14 and Fig. 2.15, where the only variable is rat plasma added to the preparation of standards. The matrix has not only had an impact on the chromatography, shifting the retention time, however also on the signal intensity, with the matrix having a suppressive effect, reducing the peak size (counts per second (cps)).

To retain tobramycin on the column for longer, the gradient was adjusted. Gradient elution is most useful for reversed phase liquid chromatography where the gradient is formed by increasing the percentage of organic solvent over time. The most effective and convenient way to alter the retention factor of a peak is to adjust the 'solvent strength' of the chromatographic mobile phase. At the beginning of the analysis, the strength of the mobile phase is low, resulting in the analyte being partitioned solely into the non-polar stationary phase. As the mobile phase strength increases, by increasing mobile phase B, the analyte will begin to partition into the mobile phase and move along the column. The rate at which the analyte moves along the column accelerates as the mobile phase strength is increased. Increasing the polarity of the mobile phase will increasingly repel the hydrophobic units of the analyte molecules into the stationary phase, thus the analyte will be retained for longer on the column. With this being the case, the percentage of organic solvent (Mobile Phase

B) was increased to 90% at 1 minute, as opposed to 0.6 minutes, and the overall gradient time was extended to 2 minutes (at a flow rate of 0.8 mL/min).

Table 2.8: *Mobile Phase Gradient (Inlet Method) adjusted to better retention of tobramycin.*

Time (min)	%A	%B
Initial	90	10
0.1	90	10
0.5	90	10
1.0	10	90
1.3	10	90
1.35	90	10
2	90	10

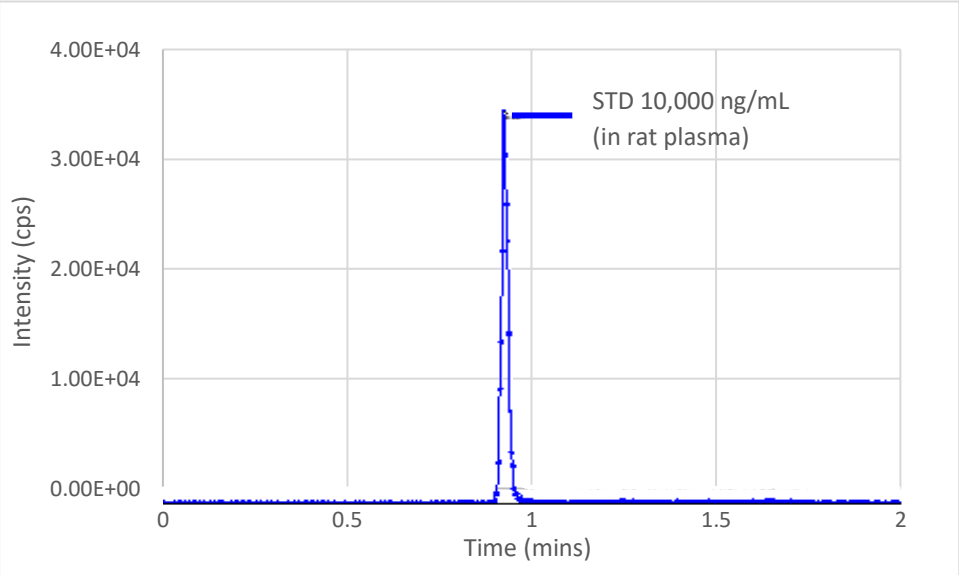


Figure 2.16: Separation of Tobramycin using the gradient as noted in Table 5.3.

Dwell Time

Dwell time is the time spent acquiring a specific MRM transition during each cycle. A very short dwell times can be used (5 ms or less), however, longer dwell times are always desirable for better s/n and sensitivity. The ideal cycle time for an MRM method is a cycle time which provides 10-15 data points across the LC peak, defining the peak apex and being optimal for accurate quantitation of samples and method reproducibility. On analysis of the peak in Fig. 2.16, a dwell time of 100 ms has achieved an adequate number of scan points.

2.5.5 Linearity Assessment

To determine the suitability of an analytical method several method parameters are required to be met. The Food and Drug Administration (FDA) [55] and the European Medicines Agency (EMA) [56], have their own documentation defining the validation of analytical methodology. The GlaxoSmithKline (GSK) Standard Operating Procedures (SOPs) are based upon these documents, and were the guidelines followed for assessment of this project method. The parameters assessed within an analytical method include but are not limited to; precision, linearity, resolution, separation efficiency, tailing factor and signal-to-noise. These method parameters were considered during this project to determine if the analytical method is suitable for sample analysis.

Linearity

The linearity of an analytical method is an assessment of the methods ability to provide results which are directly proportional to the concentration of an analyte in the sample. In an LC-MS/MS method, linearity provides confidence in a linear relationship between the analyte signal and the analyte concentration in calibration samples containing matrix. Linearity is reflected from the correlation coefficient (r) and typically, coefficients greater than 0.998 are desired. Linearity is typically determined with a minimum of five concentrations for validation procedures.

During this investigation linearity of concentration and response was determined. To achieve this, calibrants from 1 ng/mL to 10,000 ng/mL were prepared and analysed in the same manner as described in Section 2.3.2. This range was chosen to evaluate linearity across three orders of magnitude and the sensitivity of the method. The concentrations chosen were evenly spaced over the chosen range, to ensure that different parts of the calibration graph were covered.

Fig 2.17 shows the linearity of calibrants 1 – 10,000 ng/mL, where the concentrations are on the x-axis and the signals are on the y-axis. A linear fit was made through the data points using the $1/x^2$ weighting method to give the average linear equation for the line as $y = 2.35 \times 10^{-4}x - 1.09 \times 10^{-3}$, where y is the response area ratio of tobramycin/kanamycin and x is the concentration of tobramycin. The statistical measure, correlation coefficient R^2 value of 0.9983, indicates that the data is almost superimposable with the fitted regression line, however this interpretation is deceiving. Between 1 and 10 ng/mL there is no detectable

tobramycin peak, therefore the trendline is intercepting through zero, and misleadingly providing a R^2 value close to 1, for detectable concentrations of 100 – 10,000 ng/mL. The LLOQ that could be quantified reliably with acceptable accuracy and precision was 100 ng/mL showing reliable s/n, however, the LOD was 10 ng/mL.

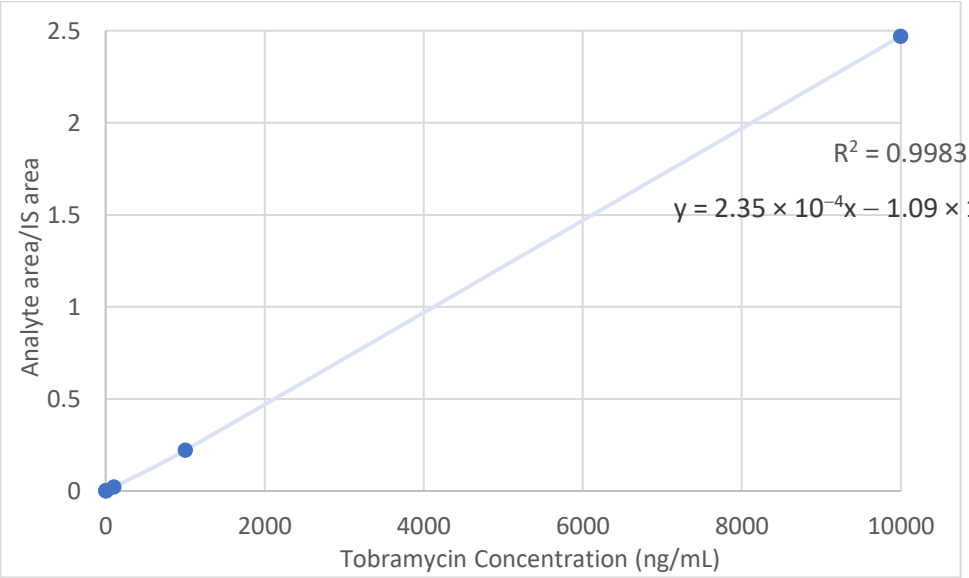


Figure 2.17: *Linearity of tobramycin calibrants 1 – 10,000 ng/mL, generated using a single data point at each concentration.*

As levels of tobramycin in biological samples are expected to be low, an LC-MS/MS method with an LLQ of 100 ng/mL is not ideal. Therefore, although the method utilised to prepare the calibration line in Fig. 2.17 is linear, it requires troubleshooting and optimisation to increase sensitivity.

Protein precipitation is a fast, straight forward extraction technique and was tested for the quantitative extraction of tobramycin from rat plasma. The extraction method used 50 µL of sample and 200 µL of IS extraction solvent. The minimal ratio recommended for this method of extraction is 3:1, organic to sample, therefore the ratio was decreased so that 50 µL of sample was extracted with 150 µL of IS. This only decreased the sensitivity, affected by a dirty sample, and increased the LLQ.

On analysis of both a blank sample, and a standard of tobramycin at 100 ng/mL, a large endogenous peak is seen directly after the peak of interest (Fig. 2.18). This peak is required to be chromatographically separated to ensure that the analyte of interest can be differentiated from endogenous components in the matrix. This peak could be potentially

supressing the signal and restricting method sensitivity. Therefore, the gradient was adjusted and extended to ensure each peak was adequately resolved. Alongside this, to concentrate and improve sensitivity of the analyte, a nitrogen blow down was conducted (explained in section 2.5.6).

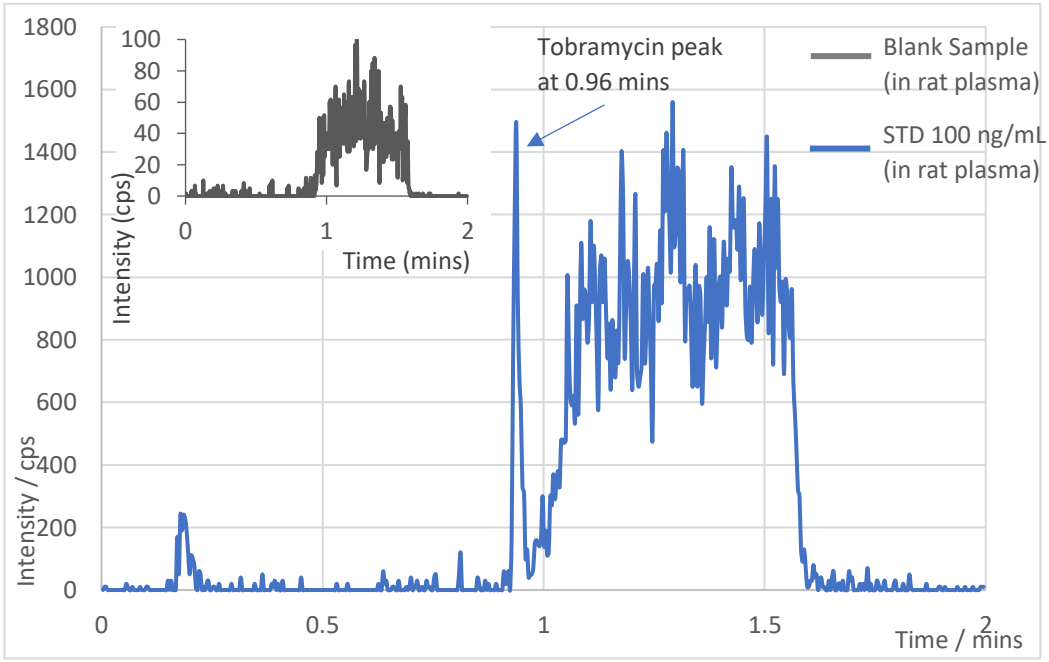


Figure 2.18: *Tobramycin peaks in a blank and standard 100 ng/mL sample highlighting the presence of an endogenous peak.*

2.5.6 Final LC-MS/MS Method

One way to improve assay sensitivity with PPT is with incorporating a nitrogen dry down step. This involves removing a volume of supernatant and evaporating the sample to complete dryness, before reconstituting in a smaller volume of a solvent of choice.

Nitrogen Blow Down

To improve LC-MS/MS assay sensitivity, often improving the sample preparation step and clean up can be beneficial. Introducing a sample with a similar composition to the starting LC conditions can aid the chromatography, retention and peak shape. Concentration of a sample provides a higher percent of analytes within a small volume of solvent for a more quantitative sample analysis.

The solvent injected may not be optimal for the LC, therefore the sample must go through a solvent exchange to be compatible with the analytical instrument. In many laboratories to prepare samples for analysis, a steady stream of gas is used to evaporate the solvent, for example pure, dry nitrogen as it is a relatively non-reactive gas. The sample is concentrated to dryness and then reconstituted in the preferred solvent as if the initial solvent is too organic, post PPT, the chromatography can be impaired. However, alongside the concentration of the analyte, any background components will also be concentrated.

The tobramycin method was updated and 150 µL supernatant was removed, evaporated to complete dryness under nitrogen at 40 °C and reconstituted in 100 µL of 50/50 Mobile Phase A/B. The reconstituted samples were then vortexed for 3 minutes prior to injection.

Columns

In section 2.1.2.1, the importance of column choice is noted. As majority of separation was completed on Phenomenex Synergi™, 50x3 mm, 4u Polar column at ambient temperature, providing limited sensitivity, a Waters Acquity™ UPLC C18 CSH column was tested for comparison. This column is too ideal for polar analytes, therefore suits a good match for tobramycin. A column temperature of 50 °C was selected due to it being in the middle of the range of the recommended operating temperatures, 20 °C to 90 °C, for the selected column. Operating at the correct column temperature enhances selectivity, lowers the solvent viscosity and increases the mass transfer rates. These changes all then allow for better chromatographic separation. Elevated temperatures will also promote basic hydrolysis of the silica support and acidic hydrolysis of the stationary phase. The effect of flow rate was also examined in the range of 0.7 to 0.9 mL/min however, it had little effect on the chromatographic peak shape and a flow rate of 0.8 mL/min was selected.

Gradient

To separate tobramycin from the endogenous peak seen in Fig. 2.18 the gradient was extended from 2 minutes to 4 minutes. Figure 2.19 shows the effects of the gradient.

Following sample injection, the mobile phase was held at 90% A for 0.5 min (the initial isocratic hold), before being decreased to 60% A until 3 minutes. From 3 to 3.1 min mobile phase A is decreased to 35 % and held until 3.5 min, within which time the final mobile

phase B composition is reached. From 3.1 to 3.5 min, a high mobile phase B%, purges and elutes any highly retained components from the column. These are components of no interest to the analytical method at hand. From 3.5 min onwards, the column is conditioned back to 90% A, the initial gradient composition. Finally, for the remainder of the 4 minutes, the column is equilibrated to ensure that it is back to starting conditions, prior to the next sample injection. This step is vital to prevent any shifts in retention leading to variability.

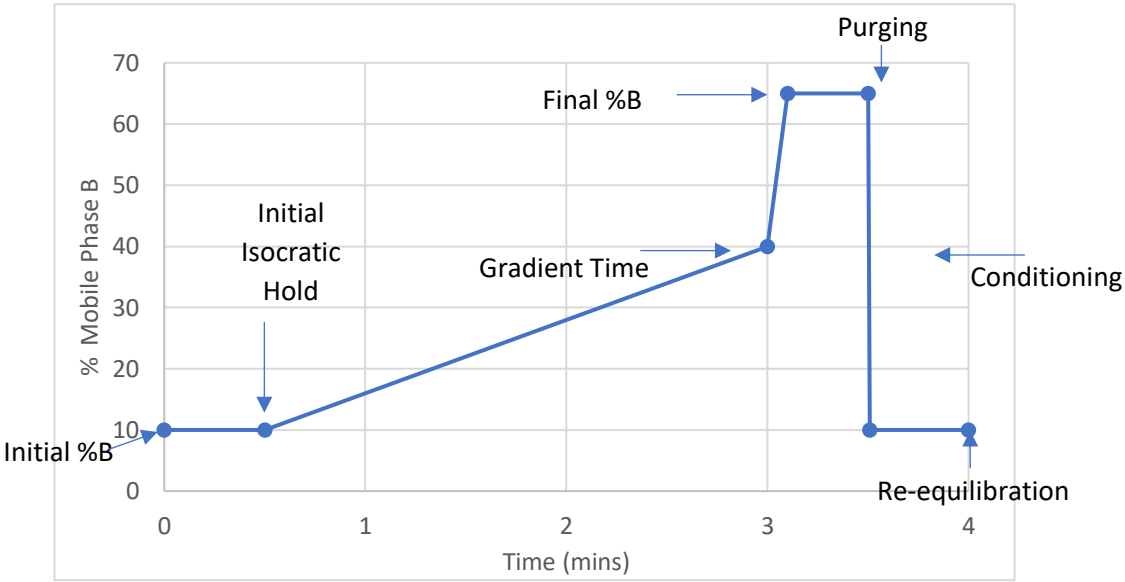


Figure 2.19: *Optimisation of gradient parameters.*

Image adapted from:
https://www.chromacademy.com/lms/sco8/Theory_Of_HPLC_Gradient_HPLC.pdf

Following the last points of method optimisation, a final extraction method was detailed for tobramycin, as noted in Table 2.9 below, to be used at validation stage.

Table 2.9: *Tobramycin Extraction Method*

Methodology	
1	Add 50 µL of sample, standard or QC into wells
2	Add 200 µL of IS working solution (ISWS) to all wells
3	Cap, vortex (5 minutes) and centrifuge (10 minutes at approximately 3000 g)
4	Transfer 150 µL of supernatant into clean tubes and nitrogen dry down
5	Reconstitute in 100 µL of 90/10 Mobile Phase A/B
6	Inject onto HPLC-MS/MS system for analysis

2.5.7 Internal Standard Optimisation

To determine an appropriate concentration of the internal standard Kanamycin, which would produce a single peak of comparable height to the HLQ of the assay, a range of concentrations were analysed before 1 µg/mL was chosen. An HLQ standard was injected, to provide a comparison with the intensity for the IS (Fig. 2.20). The ideal concentration for the internal standard is between 75 to 80% of the HLQ.

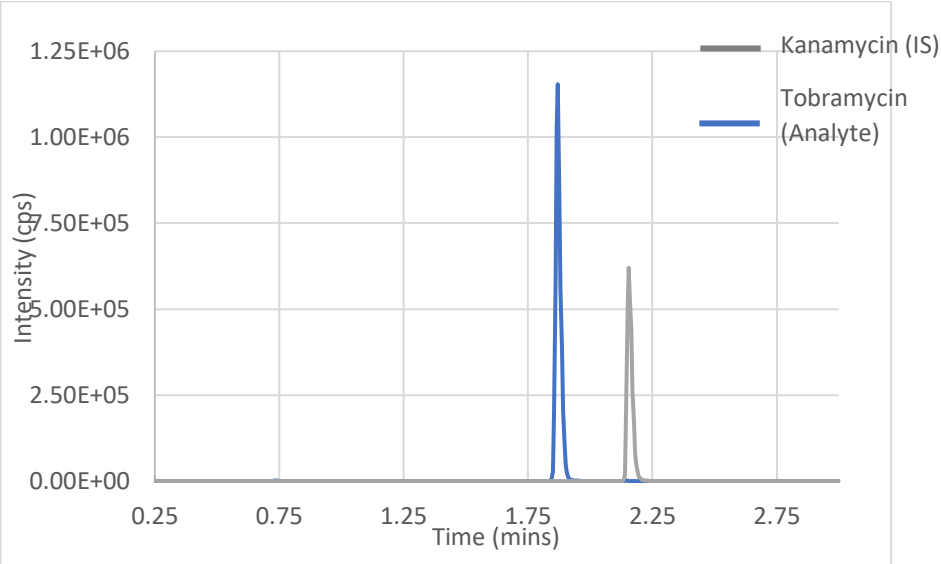


Figure 2.20: *Chromatogram depicting peak intensity between HLQ and IS.*

On analysis, there was no presence of IS in the double blank and the IS was well-resolved from the analyte peak. The IS response was of acceptable S/N ratio, a single sharp peak and with a similar retention time to the analyte of interest. Ideally, it is preferential to have an IS that co-elutes or elutes after the analyte. This provides confidence for the method as if retention time and peak response are within normal variation for that method, it can be deemed that all peaks eluting prior to the IS are also under acceptable conditions. However, as the IS is eluting near the analyte, this is acceptable too.

2.6 Conclusions & Further Work

2.6.1 LC-MS/MS Method Development Conclusions

The aim of this work was to develop a rapid, simple and sensitive method for quantification of tobramycin in rat plasma by LC-MS/MS for routine analysis in pre-clinical studies. At this point, a suitable protein precipitation extraction procedure, highlighted in in chapter one has been achieved, following appropriate troubleshooting and optimisation.

Tobramycin was extracted from 50 µL of rat plasma via PPT with kanamycin as an appropriate internal standard. Chromatography was completed on a C18 CSH (50 mm × 2.1 mm, 1.7 µm) column under gradient conditions; the mobile phases were (A) ultrapure water with 0.1 % HFBA and (B) Acetonitrile with 0.1 % HFBA. Quantitation was completed using MRM in positive mode producing transitions for tobramycin (m/z 468.3/163.1) and kanamycin (m/z 485.5/163.1). The lowest limit of quantitation (LLQ) for the method was 50 ng/mL with a linear dynamic range of 50 - 10,000 ng/mL.

2.6.2 Further Work and Validation Considerations

An LLQ has been determined using protein precipitation as an effective method of sample clean-up. The direct follow on will be to determine an appropriate calibration range and QCs and complete validation of the method highlighted for assay precision and accuracy, sensitivity, recovery, linearity, and specificity. Once this has been completed, the validated method will be used against research rat plasma samples for quantification of tobramycin.

The bioanalytical method validation (BMV) guidance for industry highlights the fundamental factors for a validation, inclusive of accuracy, precision, specificity, selectivity, reproducibility, and stability [55]. Performance of these factors demonstrates the reliability and reproducibility of the method for the intended assessment of unknown concentrations of an analyte of interest in a given matrix. The general considerations of each of these validation parameters is described below.

The future aims of a validation to these standards will provide a method assessment to understand the suitability and robustness of the LC-MSMS bioanalytical method for quantifying Tobramycin in rat plasma samples.

2.6.2.1 Precision and accuracy (P & A)

Quality control (QC) samples are used to determine the accuracy and precision of a bioanalytical method, with a recommendation to include a minimum of five replicates of QC samples at 3-5 different levels in each validation run. The QCs are prepared at concentrations as follows,

1) LOQ
2) Close to the LOQ
3) At the midrange of the calibration curve
4) Close the HLQ
5) HLQ

2.6.2.1.1 Accuracy

Accuracy describes the deviation (closeness) of the mean test results from the true concentration of the analyte [55]. Accuracy is determined by replicate analysis of typically 6 samples containing known concentrations of the analyte of interest. A freshly prepared calibration curve is utilised to determine the measured concentrations of the QC samples. The following formula is used to compare the mean measured concentrations of the QC samples to the nominal concentrations, which determines the % accuracy,

$$\%\ \boldsymbol{accuracy} = \left(\frac{\boldsymbol{mean\ measured\ conc.\ of\ replicates}}{\boldsymbol{nominal\ conc.}}\right) \times \mathbf{100}\%$$

(Equation 2.6)

For the method devised in this report, the % accuracy should be within 20% of the nominal value except at LLOQ, where it should not deviate by more than 25%.

2.6.2.1.2 Precision

Precision demonstrates the closeness in measurements of the analyte when it is at a known constant concentration, following the application of a procedure repeatedly to multiple aliquots of a single homogeneous volume of biological matrix. Precision is expressed as the coefficient of variation (CV) and measured using the equation 7.2.

$$CV = \left(\frac{SD\ of\ measured\ conc.}{mean\ measured\ conc.}\right) \times 100\ \%$$

(Equation 2.7)

Again, the CV at each concentration level should not exceed 20% except for the LLOQ, where it should not exceed 25% (following discovery acceptance criteria). Precision can be further classified as,

1) Intra-batch (within-run)
   - Precision a group of samples during a single analytical run.
   - Purpose: To determine the repeatability of the sample preparation procedure through comparison of the precision of the QCs within each run.

2) Inter-batch (between-run)
   - Precision with time e.g. different analysts or equipment
   - Purpose: To determine the reproducibility through varying the analytical method and assessing the precision of the QCs during the run.

2.6.2.2 Recovery

The recovery of an analyte is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the nominal concentration of the pure authentic analyte [55].

$$\%\ Recovery = \left(\frac{signal\ response\ of\ spiked\ samples\ before\ extraction}{signal\ response\ of\ sample\ after\ extraction}\right) \times 100\ \%$$

(Equation 2.8)

Guidelines suggest that % recovery does not need to be 100%, but the ability of the method analyte and internal standard to maintain consistency, precision, and reproducibility is the main objective [55].

2.6.2.3 Matrix Effects

Evaluation of matrix effects is a regulatory requirement during method development and validation and is defined as the effect of co-eluting compounds at the retention time of analyte, impacting LC-MS/MS ionisation and signal [57]. Often biofluids contain endogenous compounds which can interfere and cause variability in quantification [57].

Matrix effects are determined through analysis of low and high QCs prepared in six different lots of matrix, with additional evaluation in relevant patient population matrix where available [55]. Accuracy of the results for this method should be within 20% of the nominal concentration, and precision (CV) should be within 20% in all the individual matrix lots [55].

2.6.2.4 Selectivity and Specificity

The capacity of a method's selectivity is in the ability to quantify the analyte of interest in the presence of other components in the sample [55]. The analytical method must be able to unambiguously monitor the response of one analyte over other expected components such as endogenous matrix components, degradants and internal standard constituents [58]. The analyte of interest peak should be effectively resolved from all other peaks in the sample.

Selectivity is assessed through the analysis of six independent blank samples prepared in the appropriate matrix from individual donors. The blanks are analysed for the presence of interference (e.g. from metabolites and related medication), in relation to the assay LLQ.

2.6.2.5 Stability

Stability assessment of a method is required to assure reliable quantification of an analyte in a given matrix, under exact conditions for a given time [58]. The stability of the analyte is dependent on the storage conditions, chemical properties of the analyte, the biological matrix, and the storage container used. The stability of the analyte is assessed via analysis of any changes in peak separation, retention and peak shape, in comparison to a freshly prepared analyte solution.

2.6.2.5.1 Long-term

QC samples are stored at the appropriate storage conditions (temperature, storage vessels and sample volume) over a period assigned as the one expected for the authentic samples. Periodic analyses of the stored stability QCs help to monitor the stability of the analyte in the specific matrix over time.

2.6.2.5.2 Freeze/thaw (F/T)

F/T analysis ensures stability to cover when samples are frozen and thawed on multiple occasions, during reanalysis. A minimum of three cycles of F/T are advised, at 2 concentrations in triplicate. Stability QC samples are frozen at their storage temperature for 24 hours and thawed at room temperature. Once the samples are completely thawed, the samples are re-frozen again for a minimum of 12 hours under the same storage conditions. This F/T cycle is repeated two more times, and then the stability QC is compared against a freshly prepared control.

2.6.2.5.3 Bench Top (BT)

BT stability investigates stability of the analyte at ambient temperature over a period that encompasses the duration of typical sample preparation, sample handling, and analytical run time. Typically, BT is performed at 24 hours, and if instability is noted, intermediate timepoints are conducted.

2.6.2.5.4 Processed sample stability

Instability can occur in both the sample matrix as well as in prepared samples. Therefore, an assessment of the processed samples under conditions of analysis (e.g. in the autosampler or refrigerator overnight) are an important additional step of validation.

2.6.2.6 Incurred Sample Reanalysis (ISR)

ISR is conducted to evaluate the performance and reproducibility of the bioanalytical method. A reanalysis of up to 10% of the samples is performed to ensure that the results for least two-thirds of the ISR samples are within 20% of the original result for small molecule bioanalysis. This provides an additional level of confidence in the results reported.

SECTION THREE

TOBRAMYCIN ELECTROCHEMICAL ANALYSIS METHOD DEVELOPMENT

3.1 Introduction

3.1.1 Electrochemistry

Electron transfer plays a fundamental role in leading the pathway of chemical reactions. The study of electrochemistry is concerned with electron flow during chemical reactions known as oxidation-reduction (redox) reactions. Electrochemistry has become an increasingly important field of study in the last few centuries and refers to a branch of science that deals with the interactions of electron and ion phases [59]. Around the end of the 18th century, Alessandro Volta began working on one of the first batteries (Voltaic pile), which relied on electrochemical reactions in order to supply energy [59]. A few decades later, Faraday released an advancement, devising methods to understand the amount of material that is transferred during redox reactions. Significant advances in electrochemical theory and methodology have made this technique an increasingly important tool in the study of chemical systems.

3.1.2 Charge Transfer

Electrochemical reactions occur from heterogenous electron transfer between an electrode and electroactive species, where the laws of thermodynamics are maintained. In an electrochemical cell, a charge transfer reaction occurs where the passage of charge, in the form of electrons, occurs between the electrode/solution interface.

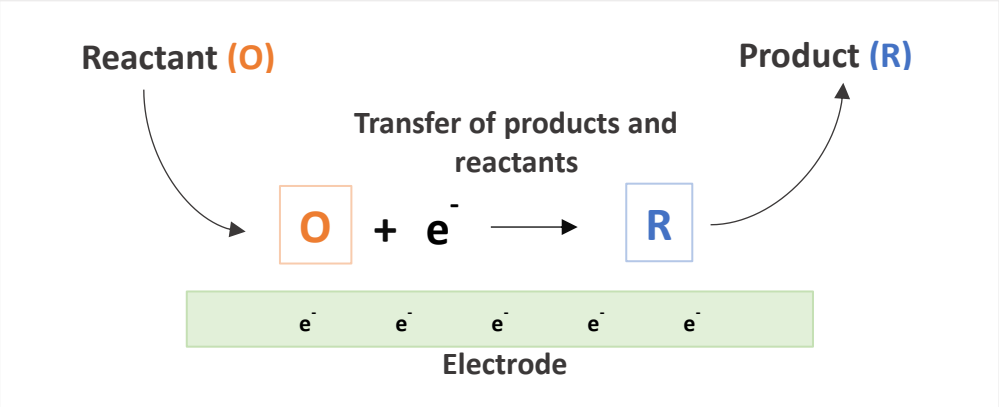


Figure 3.1: *Schematic representation of a simple electrode reaction. Adapted from [60].*

The electrode reaction usually referred to as electrolysis, typically involves a series of steps (Fig. 3.1),

1) The reactant (O) diffuses from the bulk solution to the interface (mass transport)
2) A potential is applied to the cell inducing the exchange of electrons between the electrode surface and the species in solution
3) Electrolysis occurs where the magnitude of the current is related to the change of the species in solution
4) Electron transfer occurs via quantum mechanical tunnelling between the electrode and reactant close to the electrode
5) The product (R) moves away from the electrode to allow fresh reactant to the surface

3.1.3 Fermi Level

A voltage can be applied to the electrode to control the energy of the electrons within the electrode [61]. The application of a potential is the key to driving an electrode reaction.

$$V = \frac{Joule}{Coulomb}$$

(Equation 3.1)

A volt is simply the energy (Joule) required to move charge (Coulomb) and through application of volts, electrical energy is supplied to the electrode [61]. The energy possessed by electrons in a metal electrode can be altered via applied charge. Consideration of Fermi levels can be used to understand the behaviour of electrons in metals.

Metals are comprised of electronic conduction bands, with each band possessing freely moving electrons, binding the metal cations together [61]. Therefore, metals do not possess individual well-defined electron energy levels. Instead, a continuum of levels is created with the available electrons filling the states upwards to a maximum energy level. The Fermi level directly corresponds to the energy at which the maximum electrons are. The level can be altered by applying a voltage to an electrode.

Fig. 3.2 highlights the effects of varying the applied voltage. When the Fermi Level has a lower energy than that of the Lowest Unoccupied Molecular Orbital (LUMO) of the reactant, it is thermodynamically unfavourable for an electron to jump from the electrode to the molecule [61]. However, when the Fermi level of the electrode is above the LUMO, it is thermodynamically favourable for the electron transfer to occur (reduction of O) [61].

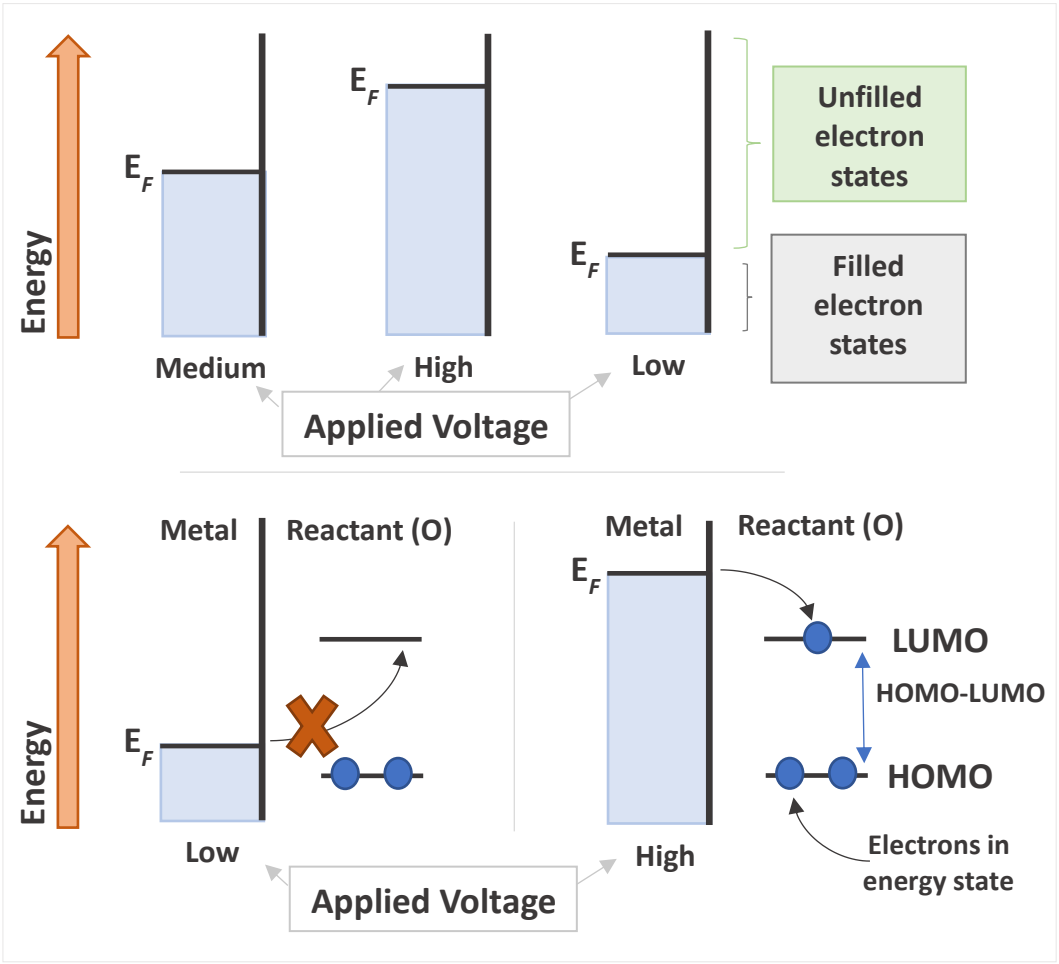


Figure 3.2: (Top) *Representation of the changes in Fermi level (E_F) at different applied voltages.* ***(Bottom)*** *Schematic representation of the reduction of a species (O) with the orbital energies (HOMO and LUMO) of a molecule. Adapted from* [60]

3.1.4 Voltammetry

Voltammetry is a branch of electrochemistry, initially developed from the 1922 discovery of polarography by J. Heyrovsky, which is employed by electrochemists for the investigation of electrolysis mechanisms [62]. There are many forms of voltammetry including linear sweep,

potential step and cyclic voltammetry with the shared characteristic of voltage application to the electrode and monitoring the corresponding current that flows through the electrochemical cell [62]. Voltammetry is an active technique as the electroactive species is either reduced or oxidised due to the potential that is applied to the electrode surface [62].

Galvanostatic and potentiometric experiments are two typical approaches to the study of heterogeneous electron transfer reactions at the electrode-electrolyte interface. For galvanostatic experiments, the electrode current is controlled in an electrolyte, and the resulting potential is measured. In potentiometric experiments, e.g. sweep voltammetry, a controlled potential is applied, and the resulting current is recorded. Potentiometric experiments are where my focus shall be, more specifically cyclic voltammetry.

3.1.5 Electrode/Solution Interface

Key interactions exist between a solid electrode and the liquid electrolyte in a voltammetry experiment under potentiostatic control. Due to the influence of the charge held at the electrode, strong interactions between the molecules in solution and the electrode surface result in the formation of the electrical double layer [60] (Fig 3.3), first introduced in the 1850's by Helmholtz.

In this model, it is proposed that as a metal electrode is dissolved for a continuous amount of time in an electrolyte, it will become increasingly negatively charged, arising from immobilised electrons at the electrode surface. This excess negative charge has an impact on the solution side of the electrode/solution interface, leading to a balance of excess positive charges in the solution. For the electrode interface to remain neutrally charged, there is a requirement for redistribution of ions close to the electrode surface, resulting in two layers of charge and a potential drop which is confined only to this region in solution.

The solvent within the electrolyte solution contributes to the potential difference across the double layer, due to its dipole nature. Water molecules are absorbed on the metal surface of the electrode, limiting the attraction for hydrated cations. Ions attracted via electrostatic forces are cushioned from the electrode surface by a layer of solvent molecules. They approach the electrode surface and form a layer balancing the electrode charge. The line drawn through the centre of such solvated cations is called the "outer Helmholtz plane". The "inner Helmholtz plane" is formed from specifically adsorbed anion

species (sometimes present in the solvent) and the solvent ions themselves, to the electrode. The diffuse layer, often called the Guoy-Chapman layer, holds excess cations or anions within which the potential changes exponentially [60].

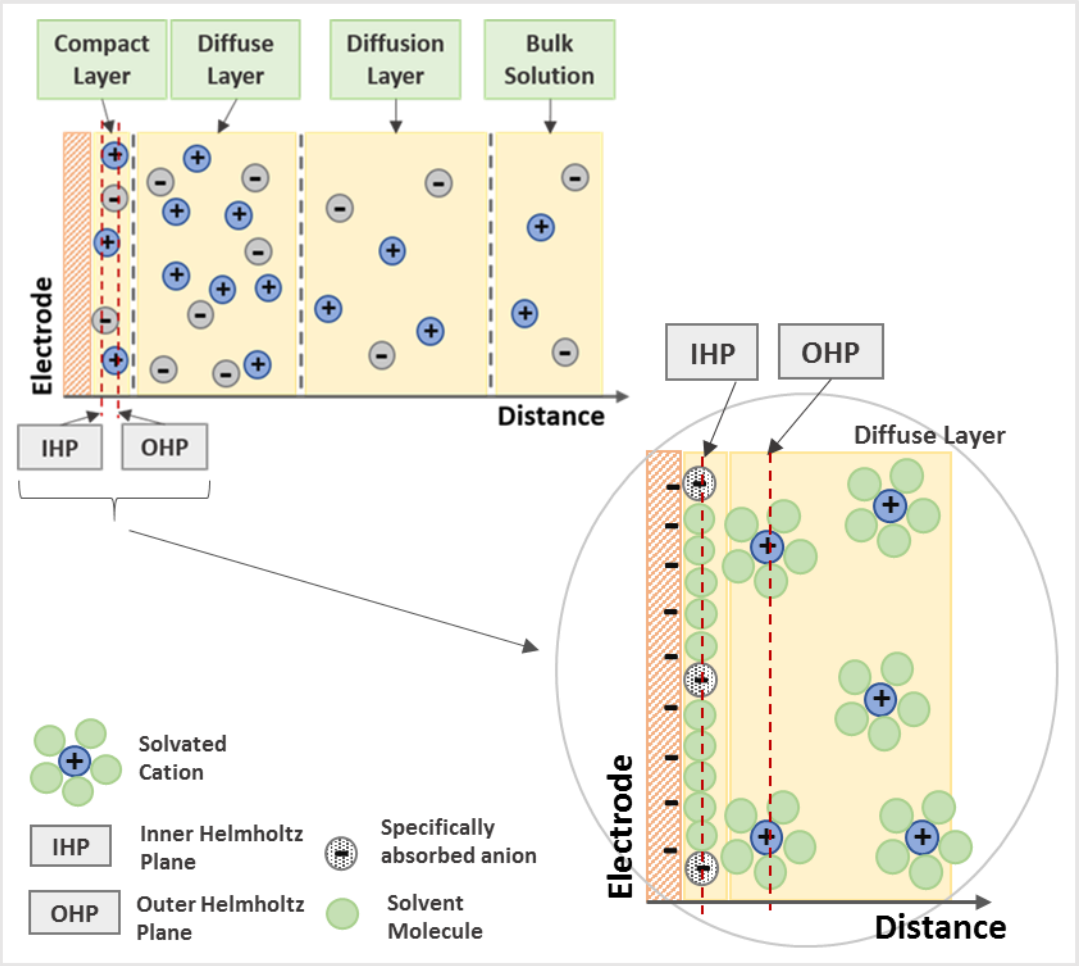


Figure 3.3: *Schematic representation of the electrode/solution interface. Adapted from* [60].

The rate of the reaction is controlled by the electrochemical pathway, with mass transport having an impact on the kinetics.

3.1.6 Mass Transport

The observed current in a voltammetric experiment is dependent upon,

1) Mass transport: The rate of material arriving at the electrode from the bulk solution
2) Charge transfer kinetics: The rate of electron transfer across the electrode-solution interface [63]

The mass transport of electroactive species (ions or molecules) within solution, from the bulk solution to the electrode surface are described by three main processes; diffusion, migration and convection [63], with each influencing the observed current and electrolysis.

3.1.6.1 *Nernst-Planck* Equation

The *Nernst-Planck* equation relates how a system will respond to a change in concentration of sample in solution, or a change in the electrode potential [61], governing mass transport.

$$\boldsymbol{J_i(x)} = -\boldsymbol{D_i}\frac{\eth C_i(\boldsymbol{x})}{\eth \boldsymbol{x}} - \frac{zi^{\boldsymbol{F}}}{\boldsymbol{RT}}\boldsymbol{D_i C_i}\frac{\eth \emptyset(\boldsymbol{x})}{\eth \boldsymbol{x}} + \boldsymbol{C_i V(x)}$$

(Equation 3.2) [60]

In this equation,

- $J_i(x)$ refers to the flux of electroactive species *(i)* at a distance *(x)* from the electrode surface.
- *Di* is the diffusion constant
- $\frac{\eth C_i(\boldsymbol{x})}{\eth \boldsymbol{x}}$ is the concentration gradient at distance *(x)*
- $\frac{\eth \emptyset(\boldsymbol{x})}{\eth \boldsymbol{x}}$ is the potential gradient
- *Zi* and C_i are the charge and concentration of species *(i)*
- *V(x)* is the velocity of movement

The three key terms expressed in the equation above relate to the three main processes of mass transfer [60]. The occurrence of an oxidation or reduction reaction at the electrode surface causes an imbalance between the presence of electroactive species in the bulk solution compared to the electrode surface; driving diffusion of analyte from one to the other [63].

According to the Nernst equation for reversible electrode reactions, the current is determined by mass transport to the electrode surface and the rate of oxidation or reduction impacting the concentration difference between the electrode/solution interface and the bulk, at a given potential [63].

3.1.6.2 Diffusion

Diffusion occurs across all solutions, arising from unbalanced concentrations of reagents [63]. In electrolysis experiments, diffusion defines the movement of electroactive species due to concentration gradients, with a higher reactant and lower product concentration in bulk solution than at the electrode. This rate of diffusion from bulk solution to the electrode can be predicted by Fick's two laws, working to quantify the processes.

Law 1,

$$\boldsymbol{J_i(x) = -D_i \frac{\eth C_i(x)}{\eth x}}$$

(Equation 3.3) [60]

Fick's first law describes how a change in concentration gradient and diffusion coefficient affects the diffusional flux of molecules. The negative sign highlights that material moves down a concentration gradient from regions of higher to lower concentrations.

Law 2,

$$\boldsymbol{\frac{\delta C_i}{\delta t} = -D_i \frac{\eth^2 C_i(x)}{\eth x^2}}$$

(Equation 3.4) [60]

Fick's second law takes into the consideration of time and variability arising as a function of time within the electrochemical cell. The rate of change of concentration as a function of time can be related to the change in the concentration gradient. Therefore, the steeper the change in concentration the greater the rate of diffusion.

3.1.6.3 Migration

Migration refers to the movement of charged particles in response to an electric field [63]. The charge of the ion, the ion concentration, the diffusion coefficient, and the magnitude of the electric field gradient can all impact migration. A change in the electrode applied potential affects solution charge migration, therefore a fixed ionic strength is maintained across the whole solution.

3.1.6.4 Convection

Convection is the movement of molecules to the electrode surface, from an imbalance of either forced or natural force and can be described by hydrodynamics. Convection introduction can be used to deliberately dominate the mass transport of reactants towards the electrode. Forced convection can occur by mechanical stirring or pumping of the solution, whereas natural convection is random in nature usually due to gradients in surface charge, temperature, vibrations or solution density [63].

3.1.7 Electrochemical Cell

The electrochemical cell (Fig. 3.4) is the most common vessel used for running electrochemical experiments, including cyclic voltammetry. A glass container is used with a cap with holes introduced for insertion of a three-electrode setup [64].

The three electrodes represent a working electrode (WE), counter electrode (CE), and reference electrode (RE), submerged into a vial where sample is dissolved with a solvent and a background ionic electrolyte.

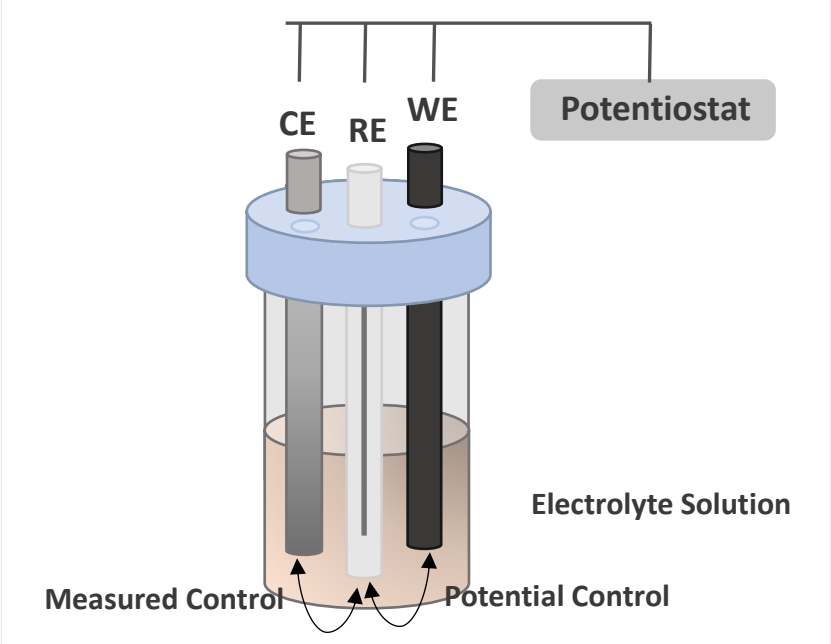


Figure 3.4: *Schematic of a three-electrode cell in solution*

The solvent used is of high dielectric constant, with increased ability to insulate charges from each other and store electrical energy within an electrical field. Example solvents of water or Acetonitrile have high polarity, and thus greater ability to stabilise charges and enable the electrolyte to dissolve, facilitating the passage of current [63].

An electrochemically inert salt, called a supporting electrolyte, is dissolved with the sample to help decrease the solution resistance (the electrical resistance between the WE and the RE) and suppress migration effects [63]. Examples of background electrolytes are sodium chloride (NaCl) in water or tetrabutyl ammonium perchlorate (TBAP) in organic solvents [63]. The mixture of the solvent and supporting electrolyte is commonly termed the "electrolyte solution".

Electrodes, made from conductive materials such as gold or glassy carbon, are connected to an external power supply, known as a potentiostat. While the current flows between the working and counter electrodes, the reference electrode is used to accurately measure the applied potential relative to a stable reference reaction.

3.1.7.1 The Potentiostat

The potentiostat applies a known external voltage, raising or lowering the energy of the electrons within the electrode and monitoring the current [62]. The potentiostat is designed such that current flows between the counter and working electrode, with negligible current through the reference. The potential difference is then measured between the reference and working electrode.

3.1.7.2 The Reference Electrode (RE)

The RE is well defined and provides a continuous stable equilibrium potential over time [61]. It is connected to the WE through the potentiostat, however current passed between the two electrodes is limited. The main purpose of the RE is to be used as a reference point, against which potentials generated from other electrodes can be measured, within the electrochemical cell [61]. For this reason, when applied potential is reported, it is most commonly denoted as "vs" a specific reference [61].

The most commonly used REs for aqueous solutions are saturated calomel electrodes (SCE), with potential determined by the reaction $Hg_2Cl_2(s) + 2e^- = 2Hg(l) + 2Cl^-$ (aq), and the silver/silver chloride electrode (Ag/AgCl), where potential is determined by the reaction $AgCl(s) + e^- = Ag(s) + Cl^-$ (aq) [61, 62]. In nonaqueous solvents, REs based on the Ag+/Ag complex are used, consisting of a silver wire in a solution containing an Ag+ salt, typically $AgNO_3$ [62].

The compound Ferrocene is frequently included in all electrochemical measurements as an IS, with reference potentials reported as "vs ferrocene" [61]. In this case, the acquired CVs are adjusted accordingly through subtracting the reference molecule's reduction potential from that of the species, such that "0" on the x-axis is the reduction potential of the IS.

3.1.7.3 The Working Electrode (WE)

The surface of the WE is usually a flat surface and is where the electrolysis of interest occurs [64]. Application of an appropriate potential, leads to the reduction or oxidation of the reactant, via mass transport of new material to the surface of the electrode and the generation of current [62]. The resulting current is what is recorded by the potentiostat. The WE is composed of redox-inert material, whereby the electrode itself does not react, and chosen according to the potential range of interest, and the requirement of surface adsorption of the compound [61]. The most commonly used WE metals are mercury, gold, platinum, and glassy carbon [62].

3.1.7.4 The Counter Electrode (CE)

The CE, or auxiliary electrode, is utilised to complete the electrical circuit, with the flow of electrons between the WE and CE being the recorded current [61]. The surface area of the CE is greater than that of the WE, which ensures that the kinetics of the reaction taking place at the CE (the transfer of electrons to or from molecules in solution quickly) do not inhibit those occurring at the WE [61]. Due to the requirement of rapid electron transfer, the most widely used CEs are non-reactive thin platinum (Pt) wires, mercury, graphite or carbon-based CEs [62], which can easily transfer electrons to or from species in solution.

3.1.8 Cyclic Voltammetry (CV)

CV is a popular, extensively used electroanalytical technique to study thermodynamic and kinetic redox processes [62]. It is often the first experiment performed in an electrochemical study [64], and useful in gaining qualitative information about electrochemical reactions for pharmaceutical compounds [63]. CV experiments are effective as they can rapidly observe redox behaviours of electroactive species over wide potential ranges [64].

3.1.8.1 Fundamentals

CV involves cycling the potential of an electrode and measuring the subsequent current produced [64]. The electrodes are immersed in an unstirred solution containing the electroactive species, and the potential of the WE is controlled vs. the RE, as explained above. The applied potential (excitation signal) to the WE is a linear scan in a triangular waveform, as shown below in Fig. 3.5.

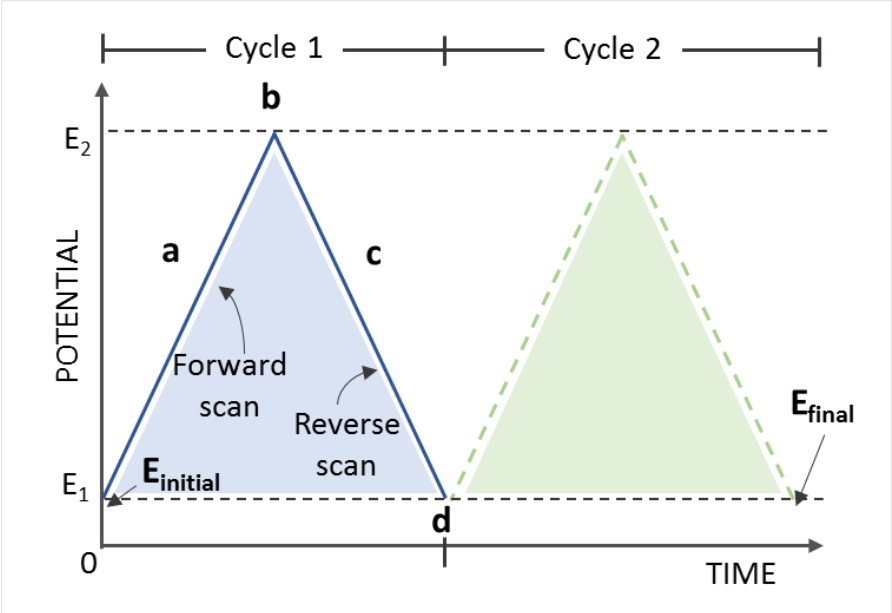


Figure 3.5: *Schematic representation of a triangular potential waveform, with switching potentials for a cyclic voltammetry experiment.*

The triangular potential is swept between two values, referred to as the switching potentials. The excitation signal (E_1) causes the potential first to scan the length of (a), whereby the electroactive species will not undergo a redox process. The potential is swept, at a rate of *v* (millivolts or megavolts per second), to the switching potential E_2, at which the

analyte undergoes a redox process. For a linear sweep, the experiment would stop here, however for cyclic voltammetry the direction of the potential is reversed from E_2 back to E_1 at a rate *v*. The current is measured at set increments across the scan and can be plotted as a function of applied potential to give the recognisable cyclic voltammogram.

3.1.8.2 Voltammograms

A voltammogram provides significant information about the electrochemical reaction occurring at the WE, and the chemical transport kinetics. The Nernst equation (given in Section 3.1.6.1) gives rise to the distinctive "duck" shape of a voltammogram, highlighting the changes due to reduction or reduction of a species (Fig. 3.6). The Nernst equation predicts that reduction of a species will occur until an equilibrium is achieved between the species ([X]) and the reduced species ([X^+]) [61].

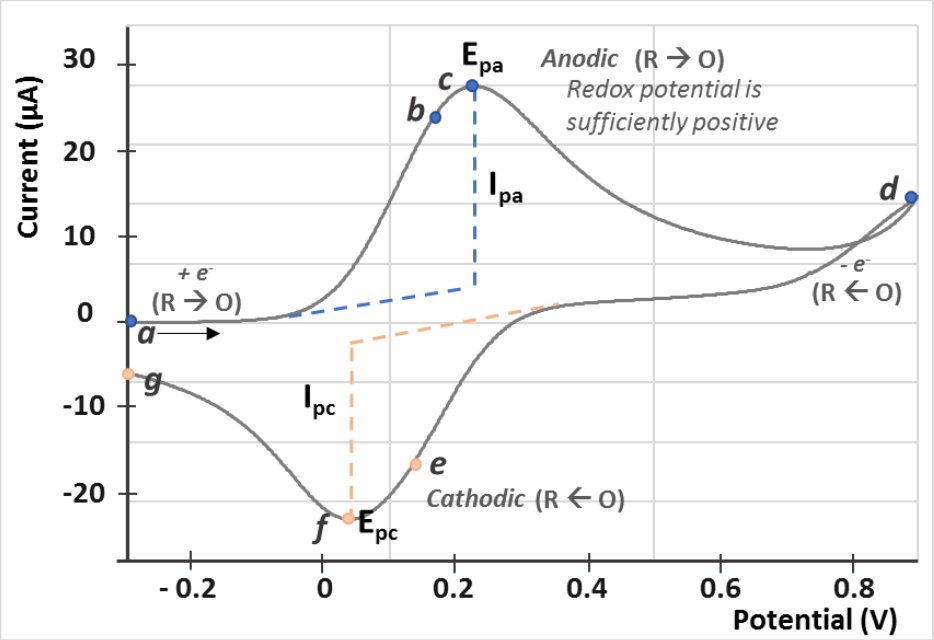


Figure 3.6: *An example voltammogram resulting from a reversible reaction of a single electron reduction and oxidation.*

As indicated by the direction of the arrow at position (*a*) in Fig. 3.6, and by the IUPAC convention [60], positive (oxidation) potentials are to the right of the voltammogram. The scan direction runs forward, positively scanning to position (*b*). The resulting current in this direction is called anodic current (I_{pa}), leading to the occurrence of oxidation whereby the [X^+] species present at the electrode surface is oxidised back to [X] as the applied potential

becomes more positive. E_{pa} (*c*) is the anodic peak potential and is reached when all the substrate at the surface of the electrode has been oxidised. Position (*d*) is where the switching potential is reached, and the scan direction is reversed.

A cathodic scan runs negatively to position (*e*), where species X, is steadily depleted and reduced near the surface of the electrode from [X] to [X^+]. The resulting current is called the cathodic current (I_{pc}), causing a reduction reaction to occur. E_{pc} is the cathodic peak potential and is reached at position *(f*) when all the substrate at the surface of the electrode has been reduced, dictated by the diffusion rate of the species [X] from the bulk solution to the electrode. A diffusion layer is created at the surface of the electrode from the volume of [X^+] species, ultimately slowing down the rate of mass transport of further [X] species to the electrode. Therefore, a drop in current from (*e*) to (*f*) is noted, resulting from a slowing rate of diffusion of [X] from the bulk solution to the electrode surface as the scan is at more negative potentials. The cycle is complete when the same potential as the cyclic scan began with is reached.

3.1.8.3 Electrochemical Reversibility

The voltammogram profile can change according to the rate of electron transfer between the working electrode and the electroactive species of interest, governed by the rate of mass transport of material to the electrode surface. The distinct “duck” shape profile can be defined as reversible (fast), quasi-reversible (intermediate) or irreversible (slow), each hosting different characteristics due to electrochemical reversibility kinetics between the electrode and the analyte [61].

Fig. 3.6 depicts the shape observed for a reversible electron transfer, highlighting that electron transfer kinetics are fast and the transfer process is easy and frequent between the electrode and the analyte of interest. For a one electron system reversible reaction the difference between the anodic and cathodic peak potentials, labelled peak to peak separation (ΔE_p) (Equation 3.5), where n is the number of electrons involved in the redox reactions) is small, at a value of 57 mV at 25°C [61].

$$\Delta \boldsymbol{E_p} = \boldsymbol{E_{p,a}} - \boldsymbol{E_{p,c}} = \frac{\mathbf{57}}{\boldsymbol{n}}\ \boldsymbol{mV}$$

(Equation 3.5) [63]

For electrochemical reversible processes, any barriers to electron transfers are low, therefore since electron transfers are fast, the Nernstian equilibrium (Equation 3.2) is established immediately upon any change in applied potential. For this reason, often electrochemically reversible processes are often referred to as "Nernstian" [61], with a ΔE_p greater than 57/*n* mV being an indicator for a not fully reversible reaction [63].

For irreversible and quasi-reversible processes, the peak separation value (ΔE_p) increases whilst the peak height decreases, as deviation from ideal reversible behaviour is observed, due to poorer electron transfer kinetics. Due to the presence of a high barrier to electron transfer more extreme negative or positive potentials are required to initiate reduction or oxidation reactions [61]. In a quasi-reversible system, a change in applied potential does not immediately trigger electron transfer. In this case the voltammogram peaks are shifted with a more positive anodic peak and a more negative cathodic peak [63]. Again, with irreversible CV systems, the cathodic and anodic peak potentials are more widely separated from one another, with each individual peak being broader and shorter in height. With some irreversible systems only one peak may be observed, due to an irreversible chemical transformation occurring at the product, resulting in a single wave with no return wave.

3.1.8.4 Randles-Sevcik equation

In a CV experiment, the features of a voltammogram that are typically monitored are peak height (I_{pa} and I_{pc}) and peak potentials (E_{pa} and E_{pc}). The scan rate can have an impact on the voltammogram observed, controlling how fast the applied potential is stepped during the potential sweep. Faster scan rates decrease the size of the diffusion layer, thus higher currents are observed with an increase in mass transport of the analyte to the electrode surface [61]. A minimum scan rate of 50 millivolts per second (mV/s) is recommended, for if the scan rate is any slower, there is a risk that analyte will be transported to or from the electrode through processes other than diffusion (for example convection).

For reversible electron transfer processes, the extent of the peak current is governed through the Randles-Sevcik equation shown below,

$$i_p = 0.446nFAC^0\left(\frac{nFvD_0}{RT}\right)^{1/2}$$

(Equation 3.6) [61]

Here the term, *n* is the number of electrons transferred in the redox event, A is the electrode surface area, D_0 is the diffusion coefficient of the oxidised analyte, C^0 is the concentration of the electroactive species in the bulk solution, ν is the scan rate, R is the universal gas constant, T is temperature and F is the Faraday constant [61].

Equation 3.6 describes how the peak current i_p increases linearly with the square root of the scan rate, providing an indication as to whether the analyte is freely diffusing in solution or if adsorption to the electrode surface has occurred [61]. A linear plot indicates that peak height is proportional to the applied scan rate and the analyte is homogenous in solution. Whilst for species which are adsorbed to the electrode surface, deviations from a linear plot would be observed [61]. Such deviations can be further distinguished to be quasi-reversible, through evaluation of ΔE_p, shifting with scan rate [61].

3.1.9 Electrochemiluminescence (ECL)

Electrochemiluminescence, also referred to as electrogenerated chemiluminescence (ECL), is a form of luminescence produced from species generated at an electrode surface, which undergo electron-transfer reactions reaching excited states that emit light [65]. Comparable to chemiluminescence (CL), ECL does not require the use of external light sources, as the production of light is through highly energetic homogenous electron transfer reactions [66].

Since the early 1960s, where ECL was first detailed by Hercules and Bard *et al.* [67], ECL has become a versatile and powerful analytical method [68], in comparison to other light-based techniques, such as CL or photoluminescence (PL) [69]. ECL combines CL and electrochemistry producing methods of high sensitivity, selectivity, and wide linear dynamic ranges for a variety of analytes, including pharmaceutical compounds [68].

A luminophore is a species capable of producing an excited-state before relaxation and the emission of a photon [69]. The presence of a luminophore, such as $Ru(bpy)_3^{2+}$ [65], is required for electron-transfer reactions to occur during ECL experiments, and the emission of light (luminescence). Luminescence in CL is initiated by the addition of one reagent to the other [68], whereby the light emission produced is obtained by highly energetic electron-transfer reactions between two reacting species (a chemical reaction). In this case, the original CL reagent cannot be regenerated, as the emitting species is an excited chemical product.

Luminescence in CL is often controlled by the careful handling of the flow rate of the reaction mixture fluid [70]. In ECL, luminescence is started and controlled by varying electrode potentials, resulting in the generation of reactive intermediates from stable precursors in the bulk solution at the surface of the electrode. The intermediate species are generated through the oxidation or reduction of precursors at the electrode surface, which undergo highly exo-energetic electron transfer reactions, resulting in the development of excited states. Upon relaxation of the generated excited states mentioned, luminescence at a characteristic wavelength, equivalent to that of the energy band gap between the ground and excited state of the luminophore is emitted [67],[65].

Unlike for CL, ECL benefits from the regeneration of ECL emitters after light emission [67], amongst many other advantages explained in Table 3.1.

Alongside the advantages, the ECL technique also hosts some limitations. ECL being an electrochemical technique requires the routine renewal of electrode surfaces, as this is key to reproducibility [66]. The electrode surface holds the reaction site; therefore, sensitivity can be affected by the quality of the light detector available [66].

Table 3.1: *Advantages of ECL*

Advantage	Reason
Electrochemical Regeneration	Some reactants, specifically the luminophore, can be regenerated at the electrode surface, allowing for a single species to participate in several ECL reactions. This factor allows for the observation of great sensitivity, from the generation of many photons during a measurement cycle [66].
Applied Potential	The ability to vary the application of potential to the WE surface allows control over the generation of the reactive intermediates and the excited states formed. This allows for control over selectivity and aids in multi-analyte detection within one sample [66].
***in-situ* formation**	The reactive intermediates or reagents required for CL may be extremely unstable, ultimately preventing analysis by traditional techniques due to their short life span [67]. ECL allows for these unstable species to be generated electrochemically through oxidation or reduction reactions occurring at the electrode surface [66]. This advantage allows for the sensitivity of ECL to be down to sub-picomolar ranges.
Light Emission	Timing and position of the light emission can be easily controlled as ECL only occurs within the diffusion layer of the electrode. Therefore, modifications in electrode size and position can be exploited to ensure the maximum number of photons produced are collected by the detector, leading to improvements in signal to noise ratio and sensitivity [66]. Controlling the time of emission can be beneficial in instances where enzyme catalyses is to occur first [67].

3.1.9.1 ECL Pathways

ECL mainly proceeds through two principal mechanisms; ion annihilation or the coreactant pathway [66]. Each mechanism follows a separate pathway, but both involve heterogenous electron transfer between the electrode and the species within the electrochemical system, to produce an emissive species [68].

3.1.9.1.1 Annihilation Pathway

Early ECL studies utilised ion annihilation pathways mostly due to its simplicity, requiring only the presence of the electrolyte, solvent and the ECL species [68]. The emission of light is controlled through potential changes at a single electrode, making the system basic and low cost. Annihilation ECL has been conserved to organic solvents as aqueous solutions are limited in potential window, preventing both oxidation and reduction from being achieved simultaneously [68].

ECL signals can be produced via an annihilation pathway involving the pulsing, or alternating, of the potential at the WE. This sweep promotes electron-transfer reactions between oxidised and reduced species, interacting to produce a ground state, followed by an electronically excited state prior to relaxation by emission [66]. The annihilation pathway is described in Table 3.2 below [71].

Table 3.2: *The annihilation pathway*

R - e$^-$	⟶	$R^{\bullet+}$	**Oxidation at the electrode surface**
R + e$^-$	⟶	**$R^{\bullet-}$**	Reduction at the electrode surface
$R^{\bullet+}$ + $R^{\bullet-}$	⟶	**R + ^{1}R***	Annihilation process leading to formation of excited state
R*	⟶	**R + *hv***	Emission of excess energy in the form of light

As described by Rizwan, *et al. (2018)* [71], the specific luminophore (R) is oxidised through the application of the appropriate potential value, generating a stable radical cation ($R^{\bullet+}$). The applied potential is then quickly switched to a value which results in the reduction of the luminophore species, generating the stable radical anion ($R^{\bullet-}$). The two radical ions annihilate producing the excited species ($^1R^*$), alongside regeneration of the ground state

luminophore (R). Relaxation of the excited species back to its ground state, sees excess energy realised in the form of light (*hv*). This emission of light has a wavelength distinctive to the energy gap between the ground and excited states [71].

Annihilation of the two radical ions (cation ($R^{\bullet+}$) and anion ($R^{\bullet-}$)) forms the excited state $^1R^*$. Depending upon the energy of the system, this formation will typically follow the singlet route (S-route) or the triplet-triplet annihilation route (T-route) [66]. In an energy sufficient system, the S-route is taken and the singlet excited state ($^1R^*$) is directly formed. However, in an energy deficient system, an intermediate triplet state ($^3R^*$) is formed first, followed by triplet-triplet annihilation to form the emission state [66].

3.1.9.1.2 Coreactant Pathway

In an annihilation pathway, described above, a double-potential step is essential to produce the excited species, however in a coreactant pathway, the applied potential to the electrode will characteristically only oxidise or reduce the species in a single potential step [65]. This single step process requires the use of a reagent (coreactant), which produces an intermediate upon oxidation or reduction, that can react with an ECL luminophore (emitter) to produce excited states [65] [67].

The coreactant pathway is now the most commonly commercially available ECL system. Most analytical instruments are based on this technology [67], to greatly enhance ECL signal, even for strong emitters such as $Ru(bpy)_3^{2+}$ [72].

A desired potential is applied to the electrode (cathodic or anodic polarity) to either oxidise or reduce both the luminophore and the coreactant species. This leads to the production of radical ions and then intermediates formed from the coreactant [71]. The intermediates decompose to produce a controlling reducing or oxidising species. This species reacts with the oxidised or reduced luminophore to produce the excited states, which upon relaxation emit light detected by the ECL detector. This is shown in Fig. 3.7, for electrochemical oxidation. An anodic current is applied initially, oxidising the coreactant and luminophore simultaneously. The coreactant radical ions formed, chemically decompose, producing a highly reducing species. Reduction of the oxidised luminophore produces the excited state, which upon emission typically refabricates the luminophore [67].

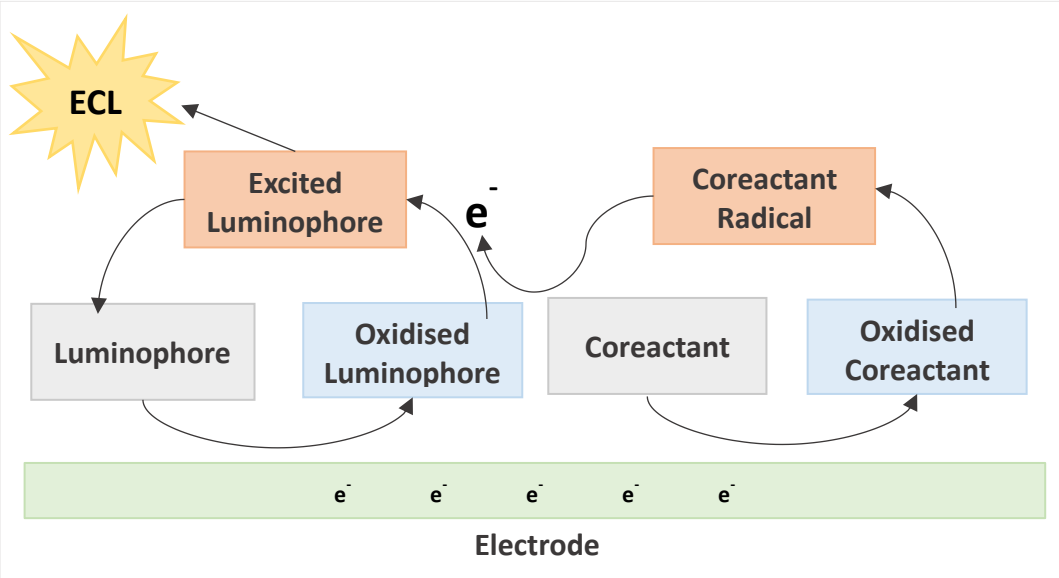


Figure 3.7: *An example of the ECL process for electrochemical oxidation. Adapted from* [73].

Depending upon the polarity of the potential, the coreactant ECL pathway can be sub-categorised into two categories (Table 3.3); one in which the coreactant is oxidised (oxidative-reductive) via the application of an anodic current or one in which the coreactant is reduced (reductive-oxidative), introducing a cathodic current [71].

Table 3.3: *Mechanisms of the coreactant pathway*

Reaction process	**Oxidative-reduction**	**Reductive-oxidation**
Initial Redox reactions	$R - e^- \longrightarrow R^{\bullet+}$ $C - e^- \longrightarrow C^{\bullet+}$	$R + e^- \longrightarrow R^{\bullet-}$ $C + e^- \longrightarrow R^{\bullet-}$
Homogeneous Chemical Reactions	$R^{\bullet+} + C \longrightarrow R + C^{\bullet+}$ $C^{\bullet+} \longrightarrow C_{red}$ $C_{red} + R \longrightarrow R^{\bullet-} + P$	$R^{\bullet-} + C \longrightarrow R + C^{\bullet-}$ $C^{\bullet-} \longrightarrow C_{ox}$ $C_{ox} + R \longrightarrow R^{\bullet+} + P$
Formation of Excited State Species	$R^{\bullet+} + R^{\bullet-} \longrightarrow R + R^*$ or $R^{\bullet+} + C_{red} \longrightarrow R^* + P$	$R + R^* \longrightarrow R + R^*$ or $R^{\bullet-} + C_{ox} \longrightarrow R^* + P$
Light Emission	$R^* \longrightarrow R + h\nu$	$R^* \longrightarrow R + h\nu$

Key:

R = luminophore, C = coreactant, C_{red} = highly reducing coreactant intermediate,

C_{ox} = highly oxidising intermediate,

P = product associated with coreactant intermediate reactions

For this ECL pathway, the choice of coreactant has a direct impact on the quality of the ECL signal. An appropriate coreactant will be easily oxidised or reduced alongside the luminophore species simultaneously, to undergo a rapid chemical reaction and form intermediates [68]. Assessment of the coreactant's stability in a wide range of solvents, solubility, electrochemical properties, kinetics, and background signal will need to be conducted [67]. Ensuring that there is no interference with the analyte of interest in solution is key [72], and that the coreactant does not quench the ECL response of the luminophore in the system.

In comparison to the annihilation pathway, the coreactant pathway has some advantages. The option of only oxidisation or reduction allows for the use of smaller potential windows, which opens the technique to be explored with aqueous systems or solvents with a narrow potential window [67]. The proportional nature of ECL intensity with coreactant or luminophore concentration, allows for the option of quantitative analysis of the luminophore, coreactant or luminophore tagged species. The coreactant is consumed during the electrochemical reaction in the pathway, leaving only the luminophore to be regenerated at the electrode [68]. This limits the number of measurement cycles which can be performed on a single sample [67].

3.2 Electrochemical Detection of Tobramycin

3.2.1 Project Aims

The focus of chapter two of this thesis will be to determine the scope of detecting tobramycin electrochemically. CV and ECL will be investigated to establish if these electrochemical techniques can be used as a qualitative screening method for the identification of tobramycin in in biological matrices. Control rat plasma will be utilised to demonstrate the limit of detection of the electrochemical method.

3.2.2 Objectives

The key points of investigation will be,

- Initial CV scans for tobramycin detection
- ECL investigation
- LLOQ assessment
- pH optimisation
- Addition of rat plasma and effects upon ECL

3.3 Materials & Methods

3.3.1 Materials

3.3.1.1 Chemicals

The electrolyte, lithium perchlorate ($LiClO_4$) was purchased from Sigma-Aldrich and prepared at 0.1M as an electrolyte solution using deionised water. Tris(2,2'-bipyridyl)ruthenium(II) dichloride hexahydrate ($[Ru(bpy)_3]^{2+}$ (Ruthenium)) was purchased from Sigma Aldrich, and was used as a luminophore, prepared at 1 mM. For ECL, 5 mm carbon SPE electrodes were purchased from GSI Technology and 5 % w/v nafion 117 from Sigma Aldrich.

3.3.1.2 Instrumentation

Electrochemical measurements were performed using a CHI 760D™ potentiostat. Either a conventional three-electrode system was used for CV or screen-printed electrodes (SPE) for ECL voltammetric measurements. For the conventional cell set up analysis was performed with a silver (Ag) wire, used as the reference electrode with a platinum (Pt) wire electrode as the counter. A 3 mm glassy carbon (GC) working electrode was used and cleaned manually following each measurement by polishing the electrode surface with 1, 0.3 and 0.05 µM alumina polish on a felt pad, in a figure of eight motion. Any residual polish was removed, and the electrode rinsed thoroughly with deionised water. When required, the electrode was sonicated for 30 minutes in de-ionised water, to effectively prevent electrode fouling.

For SPE measurements alongside ECL, GSI technology carbon paste SPE with a 5 mm carbon working electrode, carbon counter and silver reference were used. When both current and light measurements were performed simultaneously, the potentiostat was connected to a Hamamatsu™ H10723-20 photomultiplier tube (PMT). ECL measurements were collected within a light-tight Faraday cage, utilising a specifically designed holder. This worked to position the working electrode directly above the fibre optic cables coupled to the PMT, optimising collection of the maximum number of photons produced. Care was taken to ensure consistency with electrode placement to limit variability.

3.3.2 Methods

3.3.2.1 Preparation of Standards and Solutions

The presence of a luminophore (a chemiluminescent reagent) is required for ECL analysis. For this work, $Ru(bpy)_3^{2+}$ was used, prepared at 1 mM by accurately weighing out 14.90 mg of tris(2,2'-bipyridyl)ruthenium(II) dichloride hexahydrate (Sigma Aldrich) into a 20 mL volumetric flask. The flask was then prepared to volume with deionised water and covered with tin-foil to minimise light exposure and potential photo degradation of the complex.

For tobramycin standards, a stock solution at a concentration of 300 µM was prepared. For initial CV experiments, a standard of tobramycin at 100 µM was prepared in $LiClO_4$.

For ECL based experiments, dilution of a 300 µM tobramycin stock solution with $LiClO_4$ was performed to prepare a set of 1 mL calibrants, as shown in Table 3.4.

Table 3.4: *Tobramycin standards prepared by serial dilution of stock solution.*

Standard Concentration (µM)	**Volume of Previous Standard (µL)**	**Volume of Solvent (µL)**
300		
150	500	500
75	500	500
37.5	500	500
18.75	500	500
9.375	500	500
4.685	500	500
2.34	500	500

3.3.2.2 Preparation of Films

A nafion-ruthenium film was applied to the screen-printed electrodes to increase ionic conductivity. The film was prepared by taking an equal ratio of nafion stock solution (0.2% w/v in ethanol) and ruthenium solution (1 mM in ethanol). The nafion stock was prepared by dilution of the 5% w/v nafion solution to a concentration of 0.4% w/v in ethanol. A ruthenium stock at a concentration of 1 mM was prepared in ethanol by accurately weighing out approximately 3.74 mg of tris(2,2'-bipyridyl) ruthenium (II) dichloride

hexahydrate and dissolving in 5 mL of ethanol. All solutions were stored at 4 °C and ruthenium solutions were covered with tin-foil to prevent possible photo-degradation. An equal volume of the 0.4 % nafion was mixed with an equal volume of 1 mM ruthenium to prepare a final film at 1:1 0.5 mM ruthenium to 0.2% nafion. The SPE's were drop-coated with the nafion-ruthenium film by applying a 7 µL drop of the solution at the circular area and leaving to evaporate to dryness at room temperature for 2 hours.

3.4 Results and Discussion

3.4.1 Feasibility of Cyclic Voltammetry

Initial CV scans were performed to establish the peak potentials of tobramycin with the associated supporting electrolyte and the redox potential of tobramycin without the presence of a ruthenium complex. A key to the success of an electrochemical experiment can lie with the chosen electrolyte, therefore initial blank CV scans are important to determine that the electrolyte is chemically and electrochemically inert during the chosen potential window for the experiment [61]. Blank scans of 0.1 M $LiClO_4$ electrolyte were performed as shown in Fig. 3.8 (Grey) below,

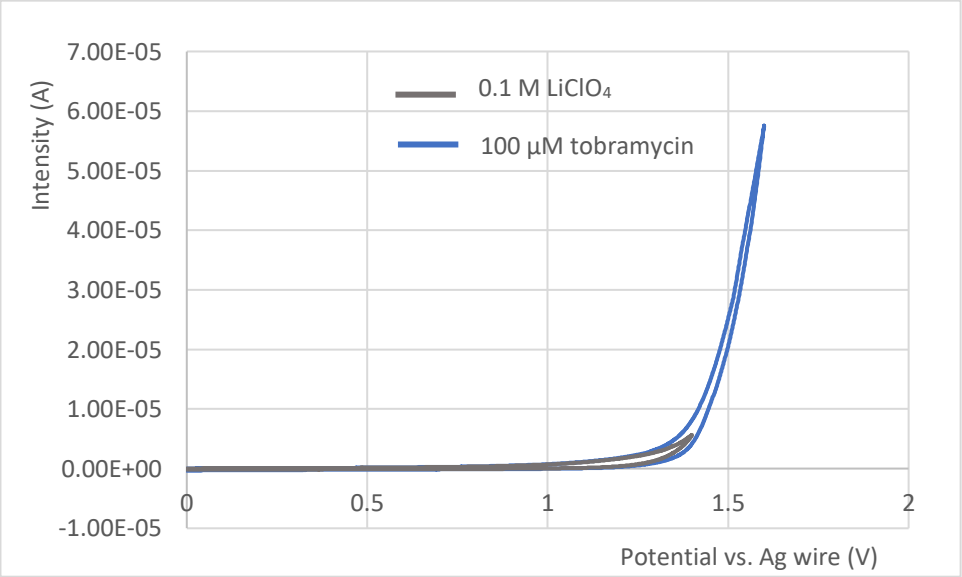


Figure 3.8: (Grey) *A typical CV of 0.1 M $LiClO_4$ across a potential range 0 V ≤v ≤ 1.4 V vs Ag.* ***(Blue)*** *CV of 100 µM tobramycin prepared in 0.1 M $LiClO_4$ across a potential range 0 V ≤v ≤ 1.6 V vs Ag. All were recorded using a GC electrode at a scan rate of 100 mvs.*

The solution showed the absence of oxidation or reduction peaks over the potential range 1.0 - 2.0 V vs Ag wire potential range, as expected [61]. This confirms that the electrolyte is inert during the potential range and the absence of any contamination in the solvent which may affect the electrochemical interpretation.

A CV scan was performed with tobramycin to understand the presence of redox activity. The scan shows that for tobramycin, CV is not feasible with the conventional cell setup utilising a three-electrode system of working, reference, and auxiliary electrodes. The redox

profile in Fig. 3.8 (Blue) shows the absence of oxidation or reduction peaks over the range 0 V ≤v ≤ 1.6 V vs Ag potential range.

3.4.2 Screen Printed Electrodes (SPE)

In a typical electrochemical cell, one major disadvantage is the frequent fouling of working electrodes from contamination and species adsorption, impacting the signal from scan to scan. As the electrochemical event occurs at the WE, it is vital for this to be a clean surface for investigation. Mechanical polishing can be used to prepare and activate electrodes, removing any species adsorbed to the WE, as explained by Elgrishi *et. al (2018)* [61]. On CV experimentation, it was apparent that tobramycin is a sticky compound, therefore strongly adsorbing to the electrode surface, preventing the re-use of electrodes for a second scan, without prior electrochemical cleaning. Increased cleaning of the electrodes did not remove the presence of a signal, indicating that tobramycin could have a high affinity for the materials of the WE.

Current methodology under development, aids with the tedious, time-consuming step of mechanical polishing, using screen printed electrodes (SPE). The disposable nature of SPE offers an advantage over conventional electrodes, eliminating any contamination issues observed due to irreversible adsorption of chemical species to the electrode coating.

SPE technology has been around since before the 1950s, utilised for printing circuit boards in the electronics industry. Since then, fabrication of the thick-film technology into an electrochemical sensing strip, has allowed the technique to be employed for the detection of biological species [74]. Miniaturisation with SPEs allows for a reduction in the sample volume required for analysis, as well as modification of the electrodes with enzymes or polymers, for more specific quantification of the analyte of interest [75]. Alongside this, SPEs offer advantages of being relatively inexpensive to produce in-house, low variability between experiments and therefore good reproducibility, and the ability to be connected to portable instrumentation [76].

SPEs are produced via layers of inks and pastes, printed upon a solid substrate [75], often an insulating ceramic or plastic surface [76], utilising the methodology of screen printing. The composition of the different inks directly impacts the sensitivity and sensitivity of the electrode [76]. A typical SPE mirrors a conventional cell setup, of a chemically inert substrate

screen printed with a WE, RE and CE [74] (Fig. 3.9). As for a conventional method, the WE is where the electrochemical activity occurs, measured by CV and ECL, with a direct correlation between the concentration of analyte and the intensity of the peak current [74].

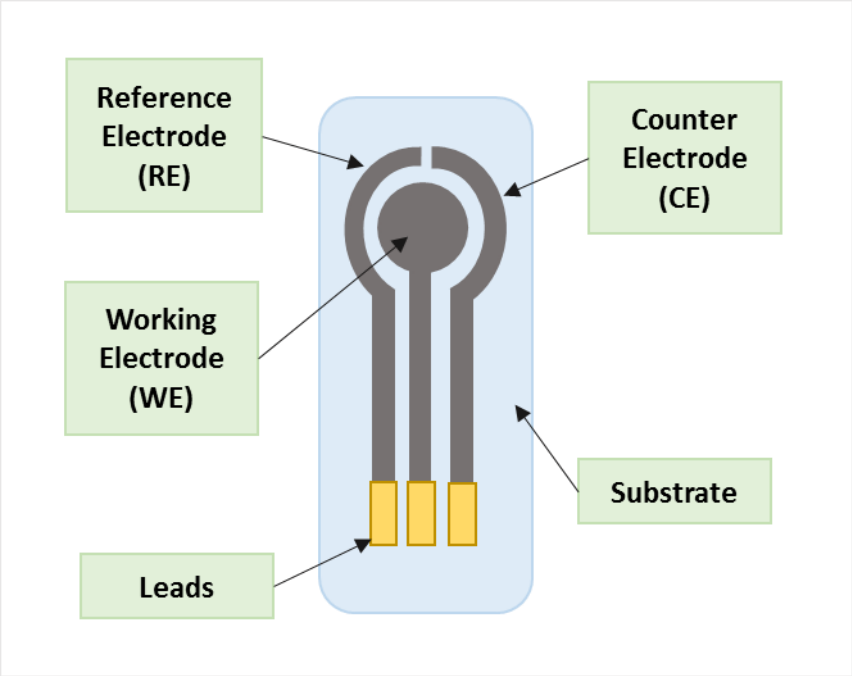


Figure 3.9: *Illustration of a screen printed electrode (SPE)* [74]

Within SPEs the WE is most often carbon, however carbon modifications do exist, such as nanotubes (CNT), or graphite [74]. Hadi *et al. (2018)* [77] reported the use of graphite carbon-based electrodes for the determination of tobramycin, benefitting from high electronic conductivity and electrocatalytic activity [77]. Like conventional cells, Ag/AgCl is used for RE material and the CE is either a platinum wire or the same material as the WE in SPEs [74].

Following the CV results, the decision was made to determine the viability of ECL for tobramycin detection with the incorporation of SPEs ordered from GSI technology.

3.4.3 $Ru(bpy)_3^{2+}$ based ECL Investigations

The ruthenium coreactant pathway is well utilised within electrochemical investigations due to advantages of ruthenium stability, sensitivity and the ability of detection across a wide dynamic range and across many analytes. The ECL of tri-n-propylamine (TPrA) and $Ru(bpy)_3^{2+}$ is popularly covered across literature in clinical testing and detection. Within this

system, the purpose of $Ru(bpy)_3^{2+}$ is to act as a luminophore, required for the generation of an ECL signal, and the TPrA is the coreactant [78] (Fig. 3.10). On application of a potential, at the electrode surface, $Ru(bpy)_3^{2+}$ is oxidised to $Ru(bpy)_3^{3+}$. The oxidised form of ruthenium can be further reduced by the oxidised form of TPrA, to produce the excited state $Ru(bpy)_3^{2+}$*. $Ru(bpy)_3^{2+}$* decays back to ground state, emitting light around 620 nm and regenerating the ECL luminophore [78]. This regeneration allows for enhanced sensitivity with ECL in comparison to chemiluminescence. Since the first reports of ECL from $Ru(bpy)_3^{2+}$ in 1972 [65], it has been used as a standard ECL emitter in both aqueous and non-aqueous applications [72].

ECL of $Ru(bpy)_3^{2+}$ can be generated via both the annihilation and coreactant pathway, however often the coreactant pathway is more commonly used due to its simplicity [79]. Well hypothesised by Rubinstein and Bard, the oxidation-reduction reaction for $Ru(bpy)_3^{2+}$ indicates that $Ru(bpy)_3^{2+}$ is regenerated at the electrode surface [80].

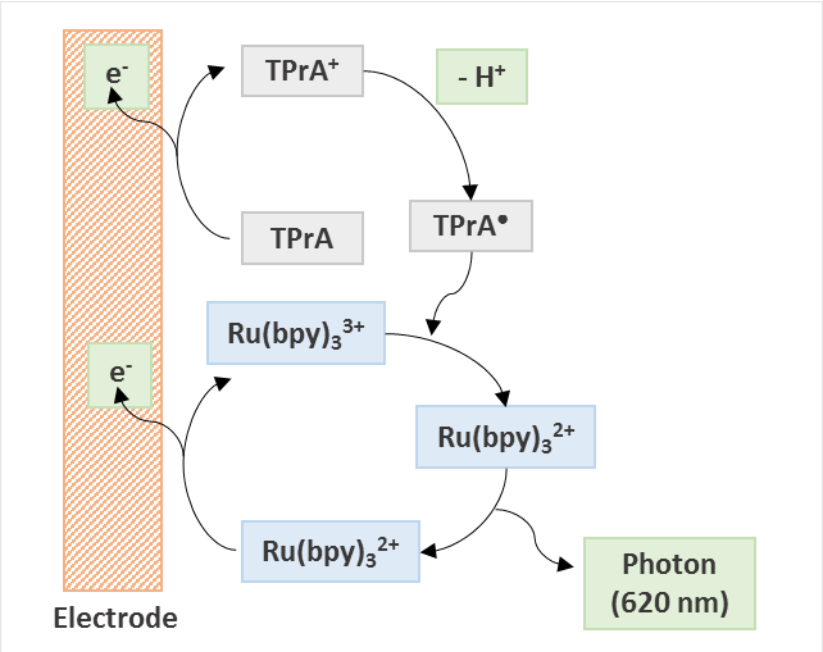


Figure 3.10: *Coreactant pathway for $Ru(bpy)_3^{2+}$ and TPrA.*

Often small molecules can be utilised as coreactants with $Ru(bpy)_3^{2+}$, due to their inability to fluoresce alone and lack of UV chromophore. Unaccompanied they possess weak electroactivity, which limits detection and highlights the need for derivatisation [79]. Therefore, for tobramycin detection, ECL is an attractive choice and alongside $Ru(bpy)_3^{2+}$, can be used to investigate the coreactant ECL activity of tobramycin.

3.4.3.1 Nafion

Nafion films have been used widely for the modification of electrode surfaces since 1980, first described by Rubinstein and Bard [80]. Nafion is a cation exchange polymer with strong chemical and thermal resistance, increasingly used as a membrane material. Due to its stability, Nafion has been widely utilised in sensors of a chemical and biochemical nature [81]. Its ion exchange capabilities allow for the easy incorporation of charged reactants such as $Ru(bpy)_3^{2+}$ into the polymer film, through electrostatic interactions between cationic $Ru(bpy)_3^{2+}$ and the anionic sulfonate groups of nafion [81]. Immobilisation of ruthenium onto the electrode surface has shown advantages over solution-based ruthenium, such as reduced reagent consumption, cost and enhanced sensitivity of the ECL system [80].

All further experimentation was acquired via the application of a nafion-ruthenium film (described in section 3.3.2.2) to the SPE, followed by a 100 µl sample. Shown in Fig. 3.11 is a representative CV profile of ruthenium with the blank electrolyte (0.1 M $LiClO_4$).

The voltammogram highlights the distinct electrochemical behaviour due to the one-electron reversible reaction of the Ru(II)/Ru(III) redox couple. In the forward scan, an oxidation peak is observed at approx. 0.89 V, depicting the formation of the Ru(III) oxidation state. The reduction peak is seen at 0.75 V as the scan direction and potential reverses, indicating the reduction to Ru(II), as described in section 3.4.3.

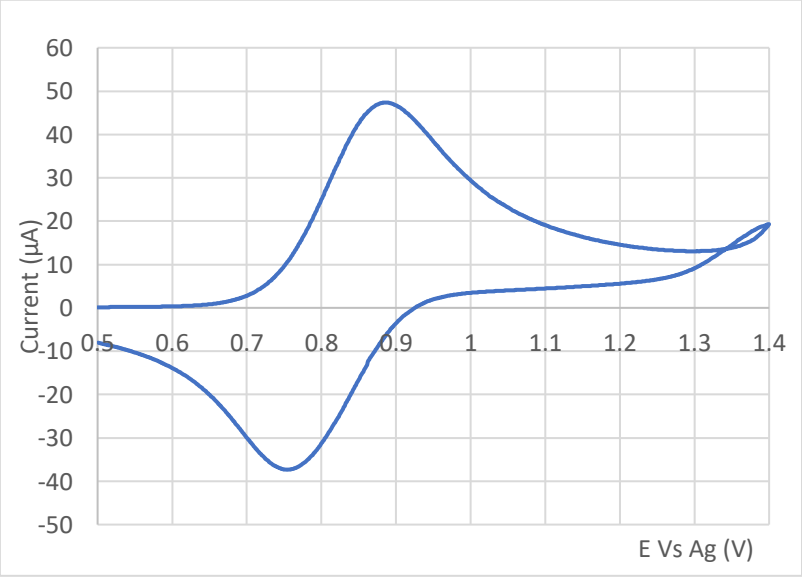


Figure 3.11: *CV of* ruthenium with the blank electrolyte (0.1 M $LiClO_4$)*, at a scan rate of 100 mV s-1 over a potential range 0.5 V ≤ v ≤ 1.4 V vs Ag on SPE.*

3.4.4 Electrochemiluminescence Analysis

Primary ECL experiments were performed to determine the feasibility of the proposed ECL methodology for the detection of tobramycin. To highlight the requirement of a luminophore for ECL analysis, an initial scan was conducted in its absence. This demonstrated the lack of ECL response without a luminophore, such as $Ru(bpy)_3^{2+}$, to provide an emission of light and the lack of induction of ECL through the pathway. When $Ru(bpy)_3^{2+}$ was present alongside tobramycin a strong ECL response was observed.

Tobramycin acts as a coreactant to the $Ru(bpy)_3^{2+}$ luminophore, facilitating the production of ECL. The presence of $Ru(bpy)_3^{2+}$ allows for the mediated oxidation of tobramycin at the electrode surface, which facilitates the ECL response. The initial ECL experimental response for tobramycin can be observed in Fig. 3.12 below, alongside the CV.

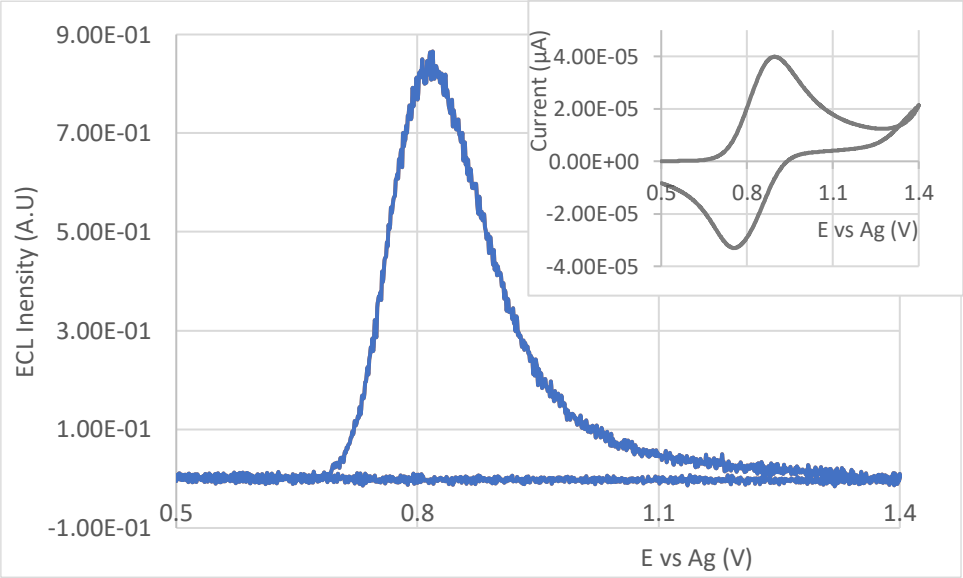


Figure 3.12: (Blue) *ECL response of 100 µM tobramycin in 0.1 M $LiClO_4$.* Collected at a scan rate of 100 mV s^{-1} across a potential range of 0.5 V ≤ν ≤ 1.4 V vs Ag, with a PMT setting of 0.7 V. **(Grey)** CV response for ruthenium.

As can be seen above, the forward scan provides an oxidation peak for tobramycin at approximately 0.8 V, confirming the emission of light being generated from ruthenium at approximately 620 nm. Between 0.5 V and 1.4 V, the proposed reaction mechanism would indicate that the oxidised form of tobramycin is acting as a coreactant to further oxidise $Ru(bpy)_3^{2+}$ at the electrode surface, peaking at 0.8 V prior to decay to ground state at 1.4 V.

The data was normalised to a baseline of approximately 0 V for the forward scan, to account for the background light detected, resulting in the starting values for ECL intensity to begin above zero. The ECL setup utilised a light-tight Faraday box and additional layers of cloth to prevent any additional light entering the unit through the door. However, even with these extra precautions, it is inevitable that some variation may occur between scans, through the need to open the door to replace SPEs.

The ECL response in Fig 3.12 depicts the ability to detect tobramycin through the modification of SPEs with a nafion-ruthenium film.

3.4.5 LLQ Assessment

To investigate the relationship between tobramycin concentration and ECL response an LLQ analysis was performed. With the theory that tobramycin behaves as a coreactant within this ECL system, a linear relationship between ECL intensity and tobramycin concentration was expected. Standards down to a concentration of 12.5 µM were analysed and detected. Samples below this concentration did not produce an ECL signal above the background. This could be due to the sensitivity of the PMT available, limiting detection below 12.5 µM.

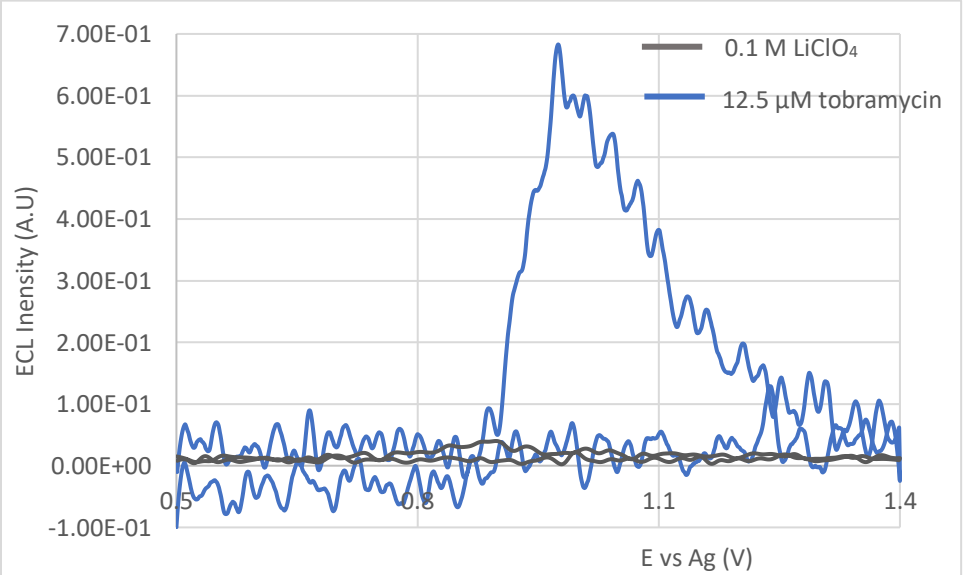


Figure 3.13: (Blue) *ECL response of 12.5 µM tobramycin in 0.1 M $LiClO_4$.* ***(Grey)*** *ECL response 0.1 M $LiClO_4$. All collected at a scan rate of 100 mV s^{-1} across a potential range of 0.5 V ≤ ν ≤ 1.4 V vs Ag, with a PMT setting of 0.7 V.*

As can be seen in Fig 3.13, the response between the background electrolyte (*0.1 M $LiClO_4$)* and a 12.5 µM standard of tobramycin is well above a 3-fold difference. This provides confidence in detecting levels of tobramycin down to this LLQ, as there is a distinction between signal to noise. The difference between signal to background also suggests that with more time, and optimised conditions such as pH, the LLQ could be reduced further.

3.4.6 pH Assessment

The production of a coreactant ECL signal is often dependant on the cleavage of a bond within the molecule and associated oxidation or reduction of the $Ru(bpy)_3^{2+}$ and the created excited species [65]. ECL signal generation and relevant intensities can also be largely impacted by the pH of the associated solutions, influencing the rate and occurrence of redox processes. pH can have an impact upon the generation of the ECL precursor species, therefore this factor was assessed.

At a pH far from the optimal, limited oxidation could be observed and thus a decrease in ECL intensity. For tobramycin rat plasma samples, the pH of the sample is expected to be approximately 8, therefore to assess the activity of the ECL setup at this pH is ideal. The effect of pH was investigated, as shown in Fig. 3.14a and Fig. 3.14b below.

The results show a linear relationship of oxidation potential and pH exist over a pH range of 6 to 8, and 9 to 10. A loss of linearity is observed between pH 8 and 9, with an optimal pH for tobramycin electrochemical analysis at pH 8. As mentioned, tobramycin is a basic substance with a pK_a of approximately 6.7 - 9.9. Therefore, increasing the pH of the electrolyte solution, favours a shift in dissociation equilibrium towards the neutral form. At a lower pH than pH 8, it is likely that the protonated form of the tobramycin compound dominates, in which form oxidation is energetically unfavourable. This theory is supported by a peak in ECL intensity at pH 8, indicating that tobramycin is at its neutral and oxidizable form, improving electron transfer kinetics.

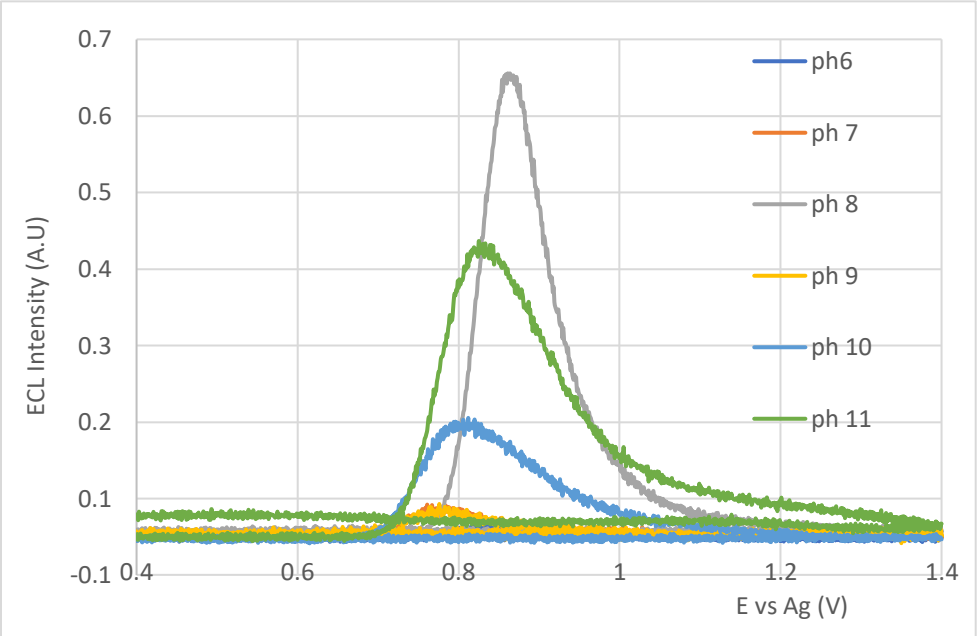


Figure 3.14a: *ECL responses for tobramycin prepared at 50 µM in 0.1 M $LiClO_4$ at pH 6 to 11* at a scan rate of 100 mV s^{-1} across a potential range of 0.4 V ≤ν ≤ 1.4 V vs Ag, with a PMT setting of 0.7 V. Figure generated using singlet data at each pH.

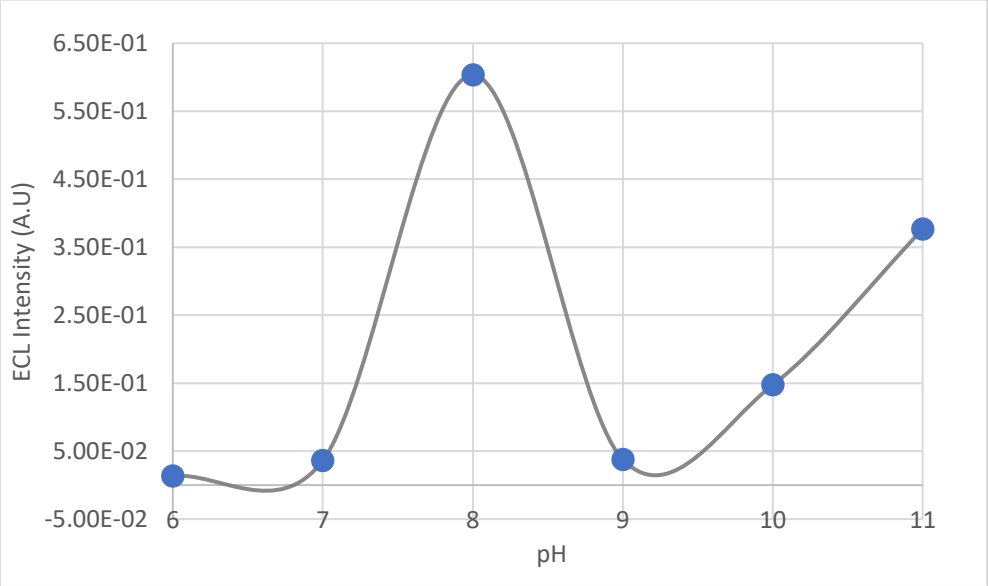


Figure 3.14b: *ECL intensity Vs pH plot for tobramycin prepared at 50 µM in 0.1 M $LiClO_4$ across the pH range 6 to 11.*

3.4.7 Linearity Assessment

To assess linearity of tobramycin concentration to ECL intensity, a range of six calibration standards from 150 to 2.34 µM were prepared, as detailed in Table 10.1. The results are shown below in Fig. 3.15.

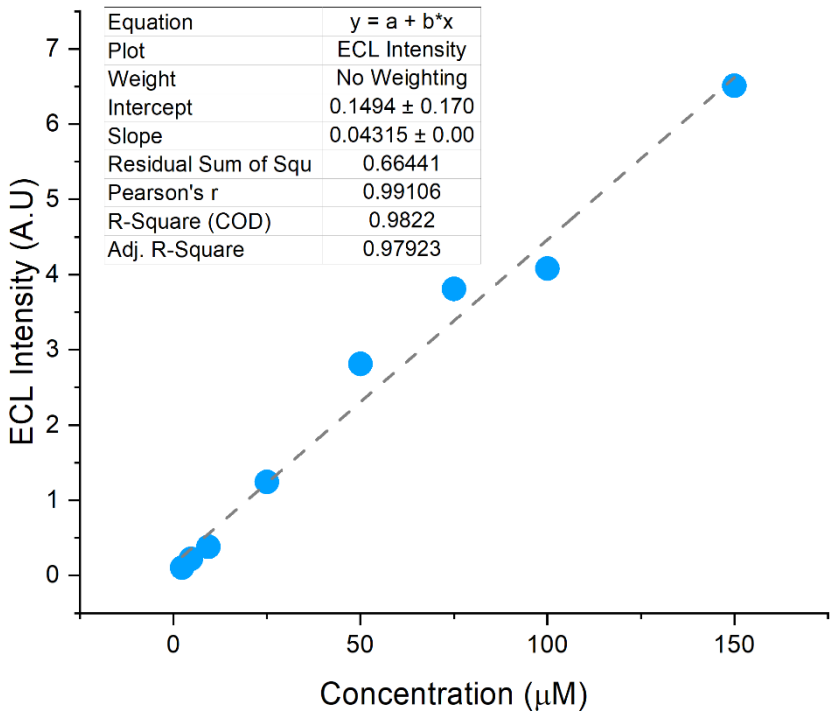


Figure 3.15: *Calibration graph for tobramycin between concentrations of 150 to 2.34 µM in 0.1 M $LiClO_4$ at pH 8. All collected at a scan rate of 100 mV s^{-1} across a potential range of 0.4 V ≤ v ≤ 1.4 V vs Ag, with a PMT setting of 0.75 V. Figure generated using a single data point at each concentration.*

The linearity plot indicates a good linear correlation between ECL intensity and tobramycin concentration, with an R^2 value of 0.982. The data obtained was with optimisation of electrolyte pH, a factor that has greatly impacted the signal allowing for an LLQ of 2.34 µM as opposed to 12.5 µM discussed previously in Fig. 3.13. The PMT setting was increased from 0.7 V to 0.75 V to maximise signal at the lower end, however during experimentation is was noted that a PMT setting above 0.75 V led to high background noise, causing the ECL response to max out.

At higher concentrations a loss of linearity was noted. Therefore, when the concentration line is truncated to 75 to 2.34 µM, removing the higher concentration standards, a better R^2 value of 0.995 is obtained.

3.4.8 Impact of Viscosity on ECL Signal

As the real samples intended for this method will be from rat, matrix was introduced to assess the feasibility of electrochemical detection of tobramycin in its presence (Fig. 3.16).

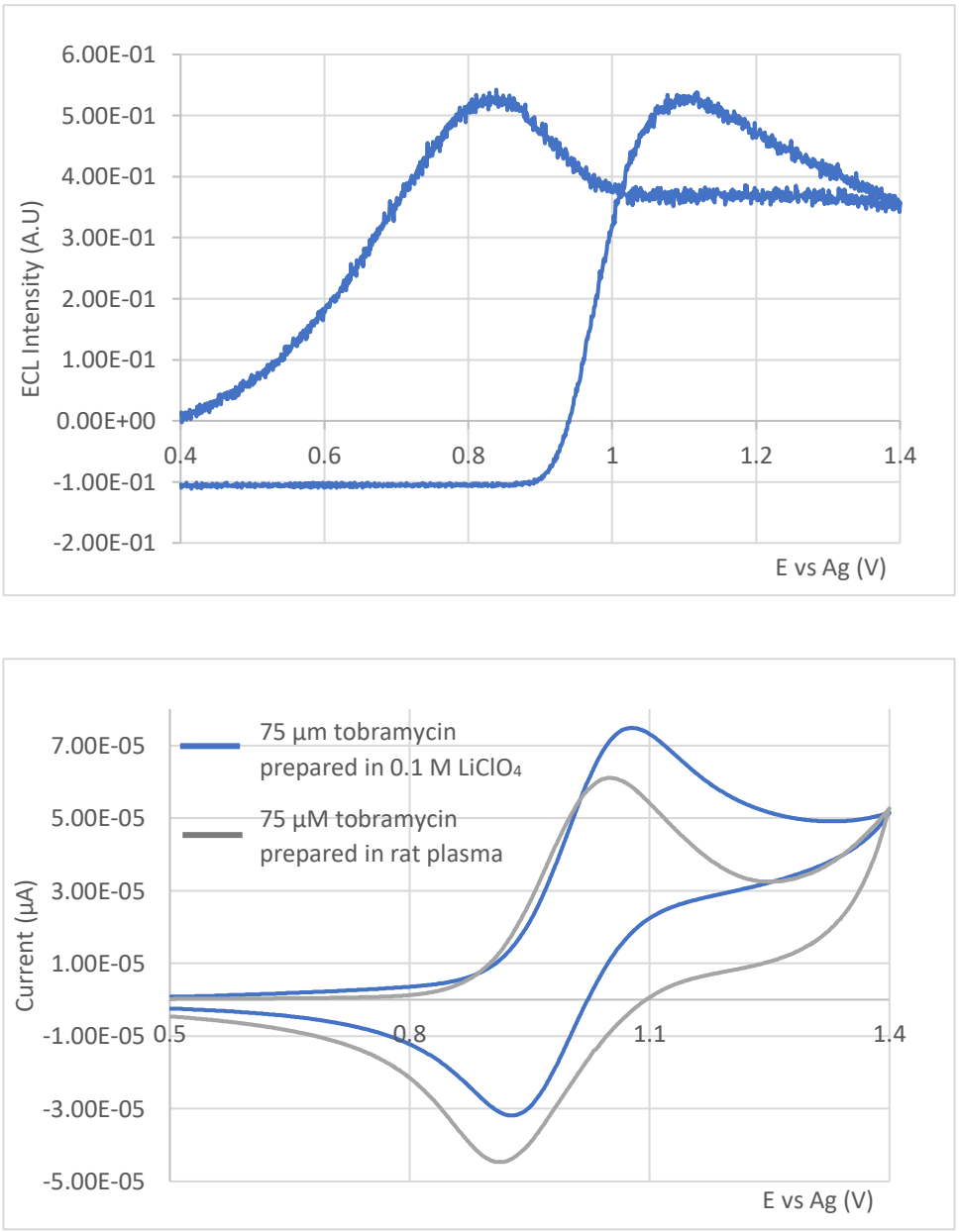


Figure 3.16: (Top) *ECL response of 75 µM tobramycin in rat plasma.* ***(Bottom) (Blue)*** *CV of 75 µM tobramycin in 0.1 M $LiClO_4$* ***(Grey)*** *CV of 75 µM tobramycin in rat plasma. All scans collected at a scan rate of 100 mV s^{-1} over a potential range 0.4/0.5 V ≤ v ≤ 1.4 V vs Ag*

The plots in Fig. 3.16 highlight the difference in redox response once matrix has been introduced to the sample. Comparison of the voltammograms depicts higher current readings for preparation in 0.1 M $LiClO_4$ in relation to rat plasma, and a distinct difference in the anodic and cathodic currents. The ECL plot has dramatically changed in shape which is most likely due to differences in diffusion of the species, away from the electrode resulting from a variation in viscosity of the solution. A rat plasma sample is a concentrated and more complex sample than blank electrolyte, possessing a different ratio of solute-solvent interactions [82]. The complexity of the sample leads to changes in viscosity and upon diffusivity. Due to the complex matrix, there is also a likely quenching of the ECL intensity as previously has been observed for pooled human serum samples [83],[84],[85]. Overall, the ECL intensity decreases with increasing solution viscosity, changing the rates of mass transport and charge transfer kinetics (explained in section 3.1.6).

3.4.9 ECL Signal for Kanamycin

As shown in chapter two, kanamycin was used as an internal standard for quantification via LC-MS/MS. As this compound behaves in a similar manner to tobramycin due to being similar in structure, electrochemical analysis was performed to highlight response. As with tobramycin and predicted, the CV response for kanamycin was also poor (Fig. 3.17).

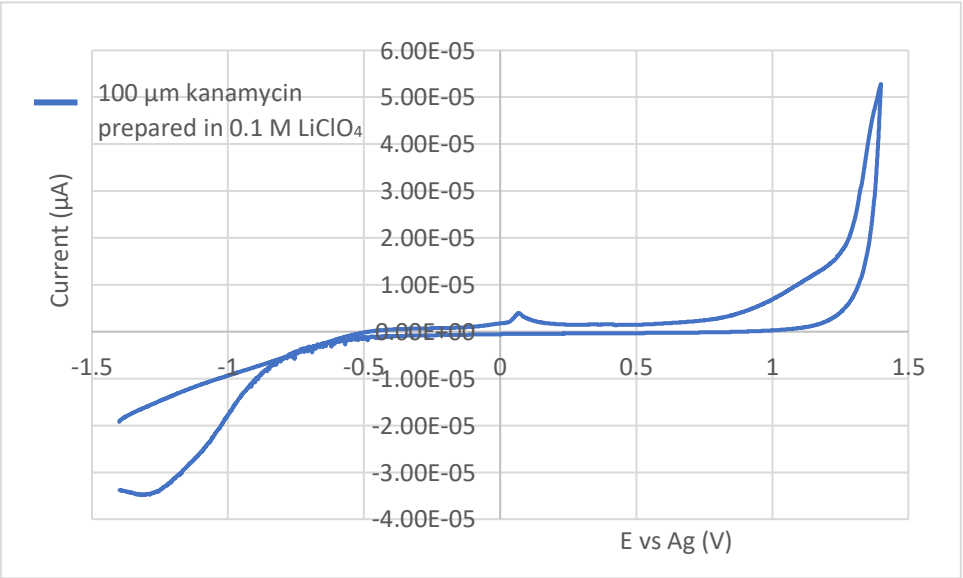


Figure 3.17: *CV of 100 µM kanamycin in 0.1 M $LiClO_4$, collected at a scan rate of 100 mV s^{-1} over a potential range -1.4 V ≤ v ≤ 1.4 V vs Ag*

A pH assessment performed with kanamycin produced results aligned with tobramycin (section 3.4.6). As shown in Fig. 3.18 below, the ECL intensity peaks at pH 8 for kanamycin, reflective of the physiological pH of the rat samples.

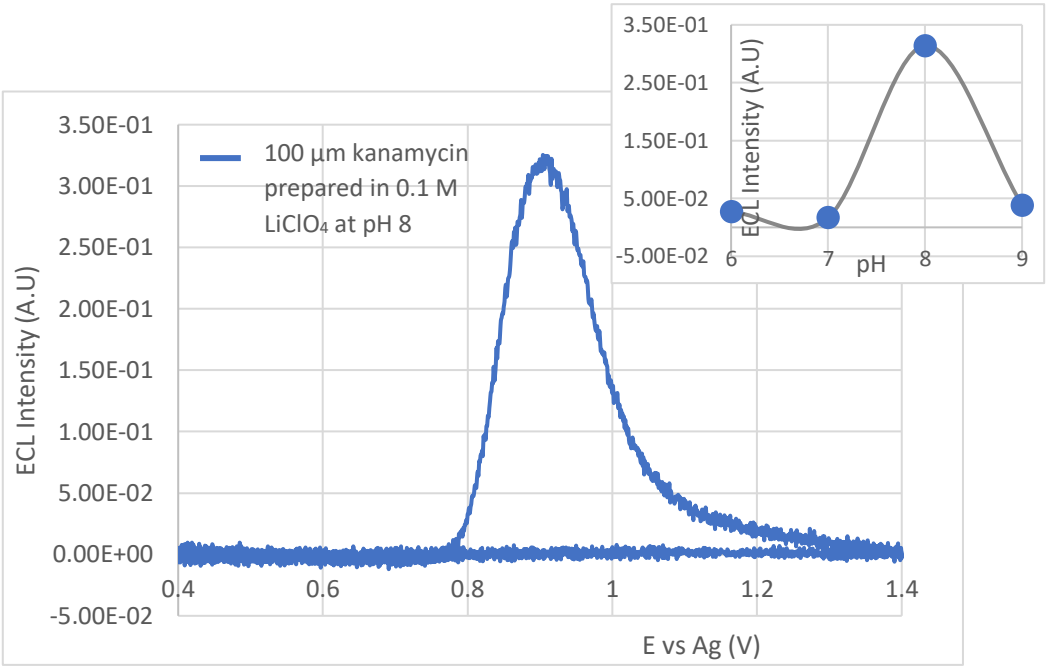


Figure 3.18: *ECL response for kanamycin prepared at 50 µM in 0.1 M $LiClO_4$ at pH 8* at a scan rate of 100 mV s^{-1} across a potential range of 0.4 V ≤ν ≤ 1.4 V vs Ag, with a PMT setting of 0.7 V. *ECL intensity Vs pH plot for kanamycin prepared at 50 µM in 0.1 M $LiClO_4$ across the pH range 6-9.*

From the ECL responses in Fig. 3.14a and Fig 3.18 it is apparent that the two compounds undergo similar electrochemical reactions to produce ECL, resulting in their ECL responses occurring around the same potential of approximately 0.9 V.

The ECL system developed and highlighted shows promise for the detection of both tobramycin and kanamycin, in a similar manner. If required, a simple pH adjustment would eliminate any possible interference with tobramycin detection, offering a pathway to distinguish between the two structurally similar compounds. Overall, although further method development and characterisation is required, the electrochemical method is a starting point for qualitative assessment.

SECTION FOUR

CONCLUSIONS FOR TOBRAMYCIN DETECTION VIA LC-MS/MS AND ECL

4.1 Conclusions & Further Work

4.1.1 Electrochemical Analysis Conclusions

The electrochemical analysis documented in section three of this report presents the ability of detecting tobramycin down to an LLQ of 12.5 µM in a 0.1 M $LiClO_4$ electrolyte sample using SPE and ECL. CV alone was not a successful system for detection. At this point, the use of SPEs modified with a nafion-ruthenium film, allowed for increased electron transfer kinetics. A linear relationship between ECL intensity and tobramycin concentration was observed, across a concentration range of 12.5 µM to 200 µM.

4.1.1.1 Further ECL Work

To increase the sensitivity and robustness of the ECL method, further optimisation will be required.

- Repeatability and reproducibility will need to be assessed, to ensure accuracy
- Investigation into the nafion-ruthenium film could increase sensitivity, by different ratios maintaining the analyte of interest close to the electrode surface by different degrees.
- Experimentation into selectivity will provide information on whether the ECL system is able to differentiate between a sample containing compounds of similar structure (e.g. tobramycin and kanamycin).

4.1.2 LC-MS/MS Vs ECL

During this project, successful detection of tobramycin was achieved via LC-MS/MS analysis without the requirement of derivatisation. ECL showed promise as a method for detection, however limited by sensitivity, as concentrations below 2.34 µM showed no detectable response. To compare the detection limits of both assays, converting to µg/µL, the LLQ for LC-MS/MS is more sensitive at 0.00005 µg/µL compared to 1.094 µg/µL for ECL. Both techniques have various steps before attaining a result, with PPT sample clean-up required for LC-MS/MS and a dilution into a supporting electrolyte for ECL. Specialised equipment is necessary for both techniques, and a level of user training. For LC-MS/MS the extraction procedure is simple to perform with reagents being readily available, which equally applies

for the sample preparation for ECL. For LC-MS/MS an understanding of both LC and MS is required to ensure suitable chromatography and MS quantification. For ECL, irregular results can be derived from problems associated with the ECL set up (e.g. the WE not being placed directly over the PMT), resulting in a lower ECL intensity. Therefore, a level of education is required to conduct both methods effectively.

4.1.3 Future Work

There is the potential to use ECL as a screening method for biosensor measurements from E-ABs, providing a quick qualitative measurement for the presence of tobramycin. Following a positive result, LC-MS/MS could be utilised to provide a more quantitative result, showing the greatest potential in sensitivity. Due to time constraints this was not possible to complete during this project, however the future work would be to receive *in vivo* rat plasma samples for quantitation and compare the result to outputs from E-ABs, utilising the two methods presented.

Even though good linear correlation is achieved with both techniques, further work can be applied. For LC-MS/MS exploring different columns suitable for polar compounds, MS detectors as only a triple quadrupole instrument and finally sample extraction methods such as SPE. SPE would provide a cleaner sample than PPT, which would present the opportunity for a more sensitive method. For ECL, as explained above, further modifications to SPEs would explore the possibility to increase detection and sensitivity. With SPE there is the potential for portable in-field analysis, which would allow the user to perform a rapid qualitative analysis themselves, a factor that could be explored by the group at the University of California, Santa Barba. Overall, a full validation for both methods would provide confidence in assay robustness for use on preclinical samples.

4.2 References

4.2.1 Report References

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