INVESTIGATION OF POST-MORTEM REDISTRIBUTION USING IN VITRO MODELS

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Abstract

In unexplained death cases it is important to be able to determine the role (if any) of the drugs detected may have played in the death. However, drug concentrations can change between the time of death and the time of the analysis of the post-mortem sample, thus the concentration of the drug detected needs to be interpreted with caution. Post-mortem redistribution (PMR) is a process that involves the passive movement of drugs after death that can lead to changes in post-mortem drug concentrations at certain sites after death. In addition, other factors that could account for post-mortem changes include the environment in which an individual is found, as certain environments could accelerate decomposition, also the circumstances surrounding the death and the length of time between the death and recovery of the body. Certain organs including lungs, liver, and heart are depots of drugs for PMR as they can have higher concentrations than surrounding sites. The bladder has traditionally not been considered a possible depot for PMR. However one study, a case report, published in Japan discussing an individual that had a PMI of nine days with higher concentrations of diphenhydramine and dihydrocodeine in the femoral vein compared to the cardiac blood, has suggested that it may be. There have been no further studies to elucidate any possible role of the bladder in the PMR process.

The aim of this thesis was to determine if the bladder is a potential site for PMR and to develop methodology to allow further study. The investigation included the influence of temperature, pH, porcine bladder degradation, and solution volume on diffusion from the bladder using *in vitro* diffusion through porcine bladder sections, whole porcine bladders and finally *in vivo* diffusion from the bladder in rat models over nine days.

This thesis looked at three methods to investigate the possible diffusion of drugs from the bladder. 1) porcine bladder sections; 2) whole porcine bladders and 3) whole rats.

The initial method used Franz Cells to determine the diffusion of rhodamine B, amitriptyline and amitriptyline's metabolite nortriptyline across the porcine bladder wall. Acceptor chamber solutions were 20 mM pH 7.4 phosphate buffer (PBS) and 20 mM pH 5 ammonium acetate (AA). Donor solutions, dependent on experiment, contained 100 mg/L rhodamine B or amitriptyline and nortriptyline in the respective solutions. Sampling was over five days. Parameters included temperature (37 °C, 20 °C, and 5 °C), pH (7.4, 5), intra-variability of porcine bladder diffusion and tissue degradation. Quantitation methods of rhodamine B (UV, Agilent, Cary 60), amitriptyline, and nortriptyline (HPLC, Dionex Ultimate 3000) were validated according to SWGTOX guidelines.

The femoral vein has been stated as the best site for sampling post-mortem blood and interpretation due to the isolation from the main viscera. However, due to the above case report suggesting redistribution from the bladder this is a possible factor that could affect this sampling site. The porcine bladder sections and whole porcine bladders were analysed to determine how much drug would diffuse through the tissue over the first 100 hrs after death, which is the initial steps in determining the likelihood of drugs diffusing from the bladder to the femoral vein. Whole porcine bladder studies used the validated UV method for rhodamine B. Experimental temperature was 20 °C. Full and half-filled porcine bladders contained

rhodamine B (100 or 200 mg/L) dissolved in pH 7.4 PBS and pH 5 AA. Triplicate analysis performed using the UV spectrophotometer at 554 nm. The in-vivo study involved catheterizing a rat and inserting silver nitrate into the bladder then securing it for Computed Tomography (CT) analysis over nine days.

There was increased diffusion of all three drugs at physiological temperature (37°C) with a peak rhodamine B concentration of 3.46 ± 2.72 mg/L (intra-bladder, pH 5), 6.69 mg/L and 6.69 ± 4.76 mg/L for amitriptyline and nortriptyline respectively (pH 7.4). The other parameters including solution pH and tissue degradation showed no significant difference for drugs diffusing through the bladder over 5 days. Concentration and volume was not a factor for rhodamine B diffusing through the whole porcine bladder tissue. There was an increase in drug diffusion over the five days with a peak concentration of 3.5 ± 1.02 mg/L (pH 7.4). The rat bladder was intact for two days, and then between 2-6 days, an opening was observed with leakage of solution. However, after day 7 this solution was not observed on the CT image. The CT data show that it is a good technique for the detection of diffusion of ions from the bladder, but would need to be further developed to look at the diffusion of larger molecular weight organic molecules.

Based on this work, methods for investigating the diffusion of drugs across the bladder have been developed and validated. The use of μCT shows promise for the further visualization of PMR to investigate not only diffusion from the bladder but also diffusion from other drug depots in the body. However, based on this work it is unlikely that the bladder is a significant source of PMR to the femoral vein, at least in PMI of less than 100 hours after death.

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Dedications and Acknowledgements

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Chapter 1: Introduction

PART 1 – The role of Forensic Toxicology in Death Investigations

1.1 Toxicology

Toxicology is generally defined as “the scientific study of poisons” (Fowler, 1995) and in the medical profession as “the study of poisonous materials and their effects upon living organisms” (Martin, 2015). Poison suggests certain compounds however, a poison can be substances assumed to be harmless including water as “the dose determines that a thing is not a poison” (Borzelleca, 2000). Toxicology applies a wide range of different areas of science to identify, quantify, and interpret analytes of interest that could be affecting human health. There are numerous areas within science and in this case, toxicology is no different with a number of types of toxicology covering numerous departments including environmental, clinical, industrial and forensic (Negrusz, 2013).

1.2 Forensic toxicology

Forensic toxicology is the application of toxicology to casework where there are issues that have legal consequences and can result as evidence in court proceedings (Council, 2010). The findings are mainly used in various court systems including criminal, civil and coroners’ court (Flanagan, Taylor, Watson, 2007; Negrusz, 2013). This area of toxicology can be further broken down into subdivisions including sport, human performance including drink driving, workplace drug testing and death investigations (Council, 2010).

Death investigation is one of the largest areas in forensic toxicology assisting in all types of cases including suicide, accidental and suspicious deaths. The forensic toxicologist works in conjunction with pathologists, analysing the biological samples sent upon resolution of post-mortem examinations. The analysis and subsequent interpretation determines if there are drugs and/or alcohol in the system of the decreased and if the resulting concentrations may have played a part in the death of the individual. Various analytical instruments and techniques are used in this process to increase the reliability and sensitivity of the results therefore; even sub-therapeutic concentrations could be identified and quantified. All this information is collated and interpreted by the forensic toxicologist resulting in conclusions on how these substances may have affected the individual before death. These conclusions would then be used as evidence as part of the case in court or other relevant investigations (Council, 2010). The history of this area can be linked with one murder case in 1833 where white arsenic was found to be the cause of death of George Bodle and identified by a developing chemical test later named the Marsh test (Hempel, 2013).

1.3 Death Investigation

The purpose of a death investigation is to determine who died, when the individual died and how an individual died (mainly to determine the manner of death and the cause of death). The manner of death is the circumstances under which the individual died and there are a number of categories including neglect, natural causes, accidental death, open verdict, misadventure, suicide and unlawful killing (Burnetts, 2015). The cause of death is the physical conditions that occur to the body resulting in the death of the individual including disease or trauma (Levine, 2013). This area of the investigation is taken over by pathologists that

carry out a post-mortem examination (PM) or autopsy on the deceased. This involves an external and internal examination of the body that could determine the cause and manner of death, time of death and collect forensic evidence for the police. Part of the forensic procedure is to collect biological samples from the body for further analysis to determine if any drugs were present and if so the concentrations of the drugs at the time of death. In deliberating causes of death, drug concentrations are a parameter considered as part of the investigation. The usual samples taken from autopsy include blood (preferentially from a peripheral site), urine, stomach contents, vitreous humour, liver, brain, and bile. Other samples that are taken in cases of advanced decomposition include bone segments, hair and muscle tissue (Cooper, Paterson and Osselton, 2010). These biological samples would be sent to a forensic toxicology laboratory for testing. The introduction of toxicology could assist in answering some questions that arise in a death investigation including: were drugs and/or alcohol present in the deceased? Where the concentrations of drugs in the range that could have caused a toxic or fatal effect (Negrusz, 2013). Forensic toxicology is essential when there are no obvious signs of death and determining the concentration of a drug at the time of death is used to distinguish between therapeutic or toxic concentrations, giving an indication if this drug was involved in the death of the individual (Negrusz, 2013). However, the toxicologist has to be guided by the case information as a number of tests could be carried out on the biological samples that would identify different types of drugs. The samples sizes can be limited therefore testing has to be selective to prevent using all the samples in case more tests are required at a later stage.

The toxicologist is responsible for every aspect of the sample from the minute it arrives in the laboratory. These responsibilities include the storage, sample preparation including chemical alterations, analysis using analytical instruments and the interpretation of the results. After initial screening tests, any drugs identified are analysed further and the results are quantified and therefore the drug concentrations are numerical. These accurate and precise results are easy to identify with validated methods. However, it is more difficult to determine the meaning of these values as there are numerous changes occurring after death that can affect drug concentrations and also there are biological differences between each person that need to be taken into consideration as these factors can also affect drug absorption and elimination (Levine, 2013; Negrusz, 2013). In addition, the toxicologist works with the police to gain information from the case reports that could put the drug concentrations into context. As a result, a number of factors could affect the interpretation of drug concentration results in post-mortem cases.

1.4 Interpretation of post-mortem toxicological results

The forensic toxicologist would use the quantified drug concentration results in the interpretation of the case. However, other information would be needed for context, as each case is different. The basic information that the toxicologist requires to enable the interpretation of drug concentrations includes identification and physical characteristics of the deceased (name, age, gender, height, weight, medical history), dates and times of the body discovery/death/autopsy, circumstances surrounding the death and the autopsy findings (Drummer, 2013). Each of these play an important role in the interpretation of the drug concentrations found from toxicological analysis. The identification of the individual allows the toxicologist to determine the

predisposition of the individual to any drugs that may have been taken due to the age, health, access to drugs and case circumstance (Drummer, 2013).

The medical history should ideally include any prescription or illegal substances that the individual was taking. In relation to the prescription drugs, if these were found during the analysis at the prescribed dose then these can be discounted as a possible cause of death. Elevated dose could also be discounted if the individual had been taking the drugs for a long period as drug tolerance could result in increasing the dosage. On the other hand, with illicit drugs including heroin and cocaine, the medical history could show that the individual had stopped using the drug for a period, possibly due to a prison term (Green, Ray and Bowman, 2014). The drug could be detected at a low dose however, that dose could be fatal as the individual had lost the original level of tolerance to the drug (Warner-Smith *et al.*, 2001).

The case history can include a wide range of information from the environment where the body was found, which could explain any decomposition present in the body, to items found at the scene that could assist in the type of screening tests carried out on the post-mortem samples (Drummer, 2013). Decomposition caused by the environment, especially if the individual was found inside in warm conditions, could explain increased drug concentrations specifically with ethanol, which can be formed post-mortem (Petković, Simić and Vujić, 2005). In addition, the small amounts of parent drug including cocaine in relation to metabolites has been stated to be due to the continual breakdown of the drug after death resulting in unreliable dose that cannot determine the amount of drug taken ante-mortem (Karch, Stephens and Ho, 1998).

The autopsy answers important questions about the condition of the body including the identification of any diseases and also, narrows down the time of death. The condition of the body can relate to decomposition and this could affect the number of samples that could be taken for toxicological analysis, as if there is a significant amount of decomposition blood and stomach contents may not be available. Knowledge of possible diseases could be useful as this could affect drug metabolism causing a build-up of drugs in the system until a toxic dose is reached, which could have contributed to or the reason for the death (Drummer, 2013). Finally, the post-mortem interval (PMI) between the time of death of an individual and the time the samples are taken at autopsy is a variable occurrence where the individual could be found immediately or left undiscovered for several days (Moriya and Hashimoto, 2001). During this time there are changes occurring within the body that can affect drug concentrations including decomposition leading to post-mortem redistribution (PMR). Sampling procedures at autopsy have been refined in recent years due to the discovery of drug concentration changes soon after death in the main organs (heart, lungs, liver) (Anderson and Jones, 1990; Prouty and Anderson, 1990). Due to close proximity of central organs to each other, there can be higher redistribution of drugs between these organs after death in comparison with peripheral sites. The heart can undergo drug movement from drug depots in the lungs and gastrointestinal tract (Cook, Braithwaite and Hale, 2000). The other drug reservoirs include the stomach and the liver, with the left side of the liver undergoing drug diffusion from the stomach (Pounder, Fuke and Cox, 1996). Therefore peripheral sampling from the femoral vein has been favoured as a sampling site that is least affected from post-mortem changes (Prouty and Anderson, 1990). However, more recent work has suggested that the popliteal vein may be an even more resilient site to post-mortem changes (Lemaire *et al.*, 2016; Lemaire, Schmidt and Denooz, 2016; Lemaire, Schmidt and Dubois, 2017).

The toxicology analysis identifies and quantifies the drugs present in the body and the metabolites of these parent drugs. The ratio of parent drug to metabolite in blood could give an indication when the drugs were taken, as when there is a significantly higher amount of parent drug than metabolite this suggests acute ingestion close to the time of death (Apple, 1989). This could suggest these drugs could have played a part in the death of the individual. In addition, any differences in the parent and metabolites potential for post-mortem redistribution would have to be taken into account (Cook, Braithwaite and Hale, 2000). There are exceptions to this rule, due to fast metabolism, in relation to heroin as this breaks down quickly to morphine then 6-monoacetylmorphne (6-MAM) and it is 6-MAM that is used as confirmation of the presence of heroin (Jones, Kugelberg and Holmgren, 2011). In addition, poly-drug use could complicate the effect of drugs with an average of 3 - 4 drugs per case (Jones, Kugelberg and Holmgren, 2011) including the mix of prescription and illicit drugs, which could result in fatal drug-drug interactions.(Luca, Patel, 2017). Pharmacogenetics have been introduced to personalise therapy for a number of drugs due to the varying metabolism that is present mainly due to inheritance, which could affect the rate of drug metabolism or elimination, resulting in potentially fatal effects of the drugs (Scott *et al.*, 2012).

However, the toxicologist may not always have all this information, which could affect the interpretation especially if the drug present was not detected in the initial screening panel. Certain compounds would need to be specifically tested for including carbon monoxide and naturally occurring insulin. The toxicologist would only know to test for these substances if the information was in the case history, possibly with evidence found at the scene (Drummer, 2013).

Other factors would need to be considered including factors arising from the death itself, as post-mortem changes occur within the body including cooling of the body, pH change and blood movement, which could result in drug concentration changes. Post-mortem results cannot be trusted in isolation due to a number of reasons including PMR (Ferner, 2008).

PART 2 – Post-mortem redistribution

2.1 Decomposition

Following death, a person will start to decompose due to the cessation of normal body processes. There are two main processes that are involved in the initial stages of decomposition of the body, which are autolysis and putrefaction (Goff, 2009). Autolysis, the first process triggered by pH decrease after death, is the breakdown of cells via the digestion of hydrolytic enzymes (Haglund and Sorg, 1996). This natural process would aid the diffusion of any drugs (or other substances in a cell) as the membrane permeability is increased (Zapata, Luna, 1989), which results from the cell membrane being damaged as a result of the lack of oxygen and other nutrients/substances that the cell requires to function (Kumar, Abbas, 2012).

After death, there is a decrease in intracellular pH, which initiates the initial process in decomposition. The main process responsible is cell necrosis, which is the death of a cell that results from prolonged injury mainly via hypoxia (lack of oxygen) and ischemia (Kumar, Abbas, 2012). During life, oxygen, glycolysis and the breakdown of creatine phosphate are necessary for the production of adenosine triphosphate (ATP) and

adenosine diphosphate (ADP); these are produced 1.5 times more frequently than lactic acid molecules (Bate-Smith, 1956). The active processes, that use ATP as energy, transfer substances against the concentration gradient (Goff, 2009). Immediately after death there is a gradual cessation of all the active transport processes, due to the depletion of oxygen and creatine phosphate that are required for the re-synthesis of ATP (Bate-Smith, 1956; Butzbach, 2010).

Putrefaction is the second process that occurs due to the increase in production of bacteria and fungi, which results from the cessation of equilibrium of the elements that sustained life (Haglund and Sorg, 1996). Putrefaction is the breakdown of tissues via anaerobic bacteria that originate from the intestine that then spread into the tissues and the bloodstream (Butzbach, 2010). This is a metabolic process that produces putrefaction gases that gradually inflate the body (Fallani, 1961; Butzbach, 2010). The putrefaction stage, which occurs after the cessation of rigor mortis (Goff, 2009) forces blood around the body starting with the heart as the blood becomes fluid here initially allowing the movement when the muscles begin contracting (Zapata, Luna, 1989; Moriya and Hashimoto, 1999). The bacteria produce gases that bloat the body and force blood through vessels possibly resulting in circulating drugs around the body (Fallani, 1961; Zapata, Luna, 1989; Yarema and Becker, 2005). One of the changes arising from decomposition that could affect the drug levels in the body is post-mortem redistribution; this phenomenon is the movement of drugs around the body after death (Pélissier-Alicot *et al.*, 2003; Drummer, 2008).

2.2 Post-mortem redistribution

Post-mortem redistribution (PMR) is a process by which the concentrations of drugs can change at specific sites after death. It is mainly thought that the redistribution is due to passive diffusion (Pélissier-Alicot *et al.*, 2003; Drummer, 2008). To allow for the diffusion process of drugs to occur, the drugs need to be released from their binding sites within the tissues, which may involve complicated processes that are involved in the autolysis and putrefaction stages of decomposition (Anderson and Jones, 1990). Passive diffusion is a process that does not require energy and is the movement of molecules down a concentration gradient (Fick, 1995). It was initially reported as a mechanism for the distribution of alcohol after death in 1940 (Berggren and Goldberg, 1940).

Initially, the realisation that there were different drug concentrations between specific organs and the blood concentrations with barbiturates was revealed in the 1960's (Curry, 1960). The potential for changes in the drug concentration with time after death in blood was determined in the 1970's with digoxin (Vorpahl and Coe, 1978) and this led onto investigating site-to-site comparisons of drug concentrations after death (Jones, 1987; Apple and Bandt, 1988). Post-mortem drug concentrations were generally found to be higher in the central area of the body (cardiac blood) than in the peripheral region (femoral blood) of the body. The ratio of these two values is known as the central/peripheral (C/P) blood ratio, which can highlight the differences in the concentrations between the two sites (Kennedy, 2010). It has been stated many times that if the concentrations are higher in the cardiac blood than the peripheral blood then post-mortem redistribution of that particular drug will have possibly taken place (Madea and Mußhoff, 2004; Yarema and Becker, 2005). However, other studies have shown that there is still PMR occurring in the peripheral sites for a number of

drugs including dothiepin (Cook, Braithwaite and Hale, 2000) and tricyclic antidepressants (Apple and Bandt, 1988; Anderson and Jones, 1990; Apple, 2011). The factor that is widely used to determine if there is possible PMR of a drug is the cardiac to femoral ratio with values above one, suggesting possible redistribution of the drug. Another simple ratio that has also been used to suggest if a drug may undergo post-mortem redistribution is the liver to femoral ratio. In this methodology a drug is considered a candidate for significant post-mortem redistribution if the liver to peripheral blood ratio is >20. The drug is considered to have no propensity for post-mortem redistribution where the liver to peripheral blood ratio is <5 (Backer *et al.*, 1990; McIntyre and Mallett, 2012; McIntyre and Gary, 2014). The same author whom suggested the liver to peripheral ratio (Iain M McIntyre) has put forward a concept of defining post-mortem redistribution as the letter "F" when calculating the propensity of a drug to undergo PMR. The F factor is based on the post-mortem to ante-mortem ratio value of a drug (McIntyre, 2014a, 2014b). However, there are issues with these assumptions as there cannot be a direct link between post-mortem and ante-mortem concentrations even with the F factor as there are far too many post-mortem changes that can occur after death to be reduced to one constant. In addition, the site dependence of drugs specifically tricyclic antidepressants result in large ratios between liver and blood and after death the release of the drugs would increase the blood concentrations to potentially toxic concentrations and the case may be wrongly interpreted as an overdose. It has been shown with numerous separate sampling sites a difference of up to 760% between venous and arterial samples can arise (Apple, 1989). Therefore, quantifying the liver and blood samples would be beneficial for case interpretation. Three mechanisms are possibly involved in the PMR process including drug release from drug reservoirs; this includes a number of organs that accumulate high concentrations of drugs. The other the processes include passive diffusion down a concentration gradient and the consideration of there being incomplete distribution at the time of death (Negrusz, 2013).

2.2.1 Factors that can affect post-mortem drug concentrations

There are many factors that can affect the concentration of a drug post-mortem, which include the characteristics of the drug, how the drug was introduced into the body, metabolism of the drug, stability of the drug, the membrane of the cell/organ in a post-mortem environment (Madea and Mußhoff, 2004) and the post-mortem synthesis of drugs (Blackmore, 1968), the conditions the body is exposed to post-mortem (temperature, pH, submersion in water), time between death and discovery of the body, circumstances surrounding the death, how the blood samples are obtained at autopsy and the storage of the samples between sampling and analysis (Anderson and Jones, 1990; Madea and Mußhoff, 2004; Kennedy, 2010). A rabbit study, in relation to PMI, showed that there can be an increase in drug concentrations immediately after death (Maskell, 2016). Also, the external parameters include environmental conditions at the site where the body was found and the conditions within the body, for instance temperature increase after death (Goff, 2009). However, drug properties and internal processes cannot determine the behaviour of a particular drug in isolation, as the length of time between death and the analysis of blood and tissue samples could affect PMR (Moriya and Hashimoto, 2001; Saar *et al.*, 2012). Other factors that could influence the amount of drug concentration changes include putrefaction, environmental conditions and trauma (Gerostamoulos *et al.*, 2012).

2.2.1.1 Properties of drugs that influence their propensity for PMR

In addition, PMR has been shown to not just affect biological tissues but affect different drugs as basic drugs show a high affinity for PMR (Moriya and Hashimoto, 1999). The physiochemical properties of a drug are factors that can affect the extent of post-mortem redistribution. These include the molecular weight, flexibility, volume of distribution (V_d), surface area, pKa, polarity, lipophilicity (log P), molecular structure, drug ionisation and the diffusion coefficient (Zapata, Luna, 1989; Hilberg *et al.*, 1999; Yarema and Becker, 2005; Giaginis, Tsantili-Kakoulidou and Theocharis, 2009). The higher the molecular weight (MW) of a drug the more hindered the drug becomes when moving through a matrix (Brodin, Steffansen and Nielsen, 2010). Molecules with a small MW, typically < 500 g/mol, are able to distribute through the body at a faster rate than larger molecules including proteins with molecular weights in excess of 100,000 g/mol (Zapata, Luna, 1989). However, molecule weight may not be as significant a factor as most of the drugs commonly used and studied have small MW including amitriptyline (277.4 Daltons), digoxin (780.9 Daltons) and trazadone (371.9 Daltons) (Negrusz, 2013). The more viscous matrices including tissues reduce the diffusion coefficient, which is the velocity of the diffusion of a drug (Brodin, Steffansen and Nielsen, 2010).

The studies above show how individual parameters affect the diffusion of drugs after death. However, the more information known about the physicochemical and structural properties of the drug, the post-mortem sampling method and the dose taken before death would assist in the determination of the extent of PMR as none of the factors taken individually could predict PMR (Giaginis, Tsantili-Kakoulidou and Theocharis, 2009). Using quantitative structure-activity relationship (QSAR) methodology it has been possible to identify the major drug constituents that are involved in PMR. This study identified those drugs with a high basic pKa value, volume of distribution above 3 Kg/L, lipophilic and flexible drugs showing favourable conditions for PMR. However, this modelling is not suitable in all cases, as 23% of the drugs included could not be predicted. (Pélissier-Alicot *et al.*, 2003, 2006; Drummer, 2008; Giaginis, Tsantili-Kakoulidou and Theocharis, 2009).

The drugs most likely to undergo this redistribution are small, weak and basic lipophilic drugs with a large volume of distribution (V_d) (Pélissier-Alicot *et al.*, 2003, 2006; Drummer, 2008). The term volume of distribution is used to describe the distribution of drugs to the organs and is defined as the amount of drug in the body divided by the plasma concentration when distribution is at equilibrium (Hilberg *et al.*, 1999). Previous work has shown that drugs with a volume of distribution (V_d) above 3-4L/Kg are more likely to undergo redistribution (Hilberg *et al.*, 1999; Pélissier-Alicot *et al.*, 2003; Rodda and Drummer, 2006; Drummer, 2008). This has been demonstrated for a number of drugs including methadone (Milroy and Forrest, 2000)**,** fluoxetine (Pohland and Bernhard, 1997) and a number of tricyclic antidepressants including amitriptyline (Hilberg *et al.*, 1993) and nortriptyline (Moriya and Hashimoto, 1999) with V_d values of 4-7 L/kg, 20-42 L/kg, 6-10 L/kg and 20-57 L/kg respectively (Baselt, 2008). However, certain drugs that have a small V_d have demonstrated the ability to redistribute post-mortem including clozapine (2-7 L/kg), midazolam (1-3 L/kg), phenytoin (0.5-0.8 L/kg), triazolam (1.1-2.7 L/kg) and zolpidem (0.5-0.7 L/kg) (Baselt, 2008; Ferner, 2008).

Other characteristics could be the reason for redistribution of the less lipophilic drugs, these parameters are included when determining whether a drug can redistribute and to what extent. These characteristics include the partition coefficient, pKa and the pH of the surrounding environment. In addition, the pharmacokinetic stages drugs undergo may also need to be taken into account as there could be modifications during the absorption, distribution, metabolism and the elimination stages (Pélissier-Alicot *et al.*, 2003; Ferner, 2008). Incomplete absorption and distribution could lead to higher drug concentrations after death, specifically when death occurs shortly after ingestion of the drug creating a reservoir in the stomach of a high dose of the drug that would distribute even after death shown by the instillation of 3,4-Methylenedioxymethamphetamine (MDMA) into the stomach and trachea (Letter, Clauwaert and Belpaire, 2002). Metabolism occurs at different rates depending on the drug and in the case of heroin, which breaks down quickly to the metabolites including 6-MAM and morphine, which would give underestimated concentrations of the drug from post-mortem samples (Maskell, 2016). In addition, metabolism and elimination have similar issues. In relation to metabolism one of the liver enzymes that breakdown xenobiotic drugs is CYP2D6. However, some individuals have either reduced amount or no function of this enzyme and this would reduce the rate in drug metabolism. Therefore, even after a therapeutic dose the accumulation of drugs in the system could result in toxic concentrations (Droll, 1998; Abdel-Rahman, 2002). Elimination can be disrupted when there is organ failure, in the case of renal failure a therapeutic dose of gabapentin could accumulate in the body resulting in a high blood concentration (72 mg/L) when analysed post-mortem (Winecker, 2015). Also, a human study has shown drugs with a large V_d including fluoxetine, citalopram and olanzapine have demonstrated little redistribution after death comparing samples taken at the mortuary with autopsy samples (Gerostamoulos *et al.*, 2012). As a result, this shows that this is not the only parameter to determine the redistribution capability of a particular drug.

The pKa is the dissociation constant (Pélissier-Alicot *et al.*, 2006) and is the pH of a drug, at which it is 50% ionised (or 50% unionised). Using the Henderson-Hasselbalch equation and the pKa of a drug the ionisation state of a drug at a specific pH can be determined. For example if a drug (atenolol) with a pKa of 9.6 (Manallack, 2011) was present in a solution at physiological pH (7.4) would be ionised and therefore able to pass through cell membranes to redistribute.

The lipophilicity determines the ability of the drug to pass through the cell membrane. The more lipophilic a drug is the more it will accumulate into the tissues, which creates the concentration gradient needed for passive diffusion to occur after death (Yarema and Becker, 2005; Rodda and Drummer, 2006). Lipophilicity has been shown to play a part in determining the ease of transport of a drug through a membrane via passive diffusion however, it is not the main factor that could affect the overall PMR process (Giaginis, Tsantili-Kakoulidou and Theocharis, 2009). Studies have been carried out showing preferential accumulation of basic drugs dependant on the lipophilicity, a basic drug (quinine) accumulated in the lungs can be displaced by a more lipophilic basic drug (imipramine) due to competitive binding sites within the lungs (Hisahiro, Katsuhiko, 1989). The same was found with two antidepressants resulting in clomipramine displacing a carbon-11 labelled cyanoimipramine, which could lead to toxic concentrations in the bloodstream (Suhara *et al.*, 1998).

2.2.1.2 Diffusion Coefficient

The creation of a concentration gradient initiates the movement of a drug from a highly concentrated site to a site of low concentration. This can be measured using an equation named Fick's first Law after Adolf Fick, a German physiologist who suggested that diffusion is proportional to the concentration gradient (Fick, 1995). Mass transport of molecules in a solution has a measurement of fluxes and a flux is the mass or number of molecules moving through a given cross-sectional area over a specific period. Fick's law describes a flux along a concentration gradient in single plane overtime for a solute with a defined diffusion coefficient. Fick's law describes the passive diffusion process across a membrane using the equation:

$$\frac{dC}{dt} \propto \frac{A.D(C_1 - C_2)}{h}$$

C_1-C_2 = Concentration gradient of unionised form of the drug (mg/ml)
A = Surface Area (cm^2)
D = Diffusion Coefficient (cm^2/sec)
h = Thickness (cm)

Flux studies are mainly used in the analysis of the transport of drugs through tissue models. A flux could only occur if there is a concentration gradient, for example if there were insignificant movement of ions across the membrane there would be no concentration gradient and the flux would be constant (Brodin, Steffansen and Nielsen, 2010).

The diffusion coefficient is a constant that is related to the properties of a specific molecule in a specific solvent and indicates the velocity of the diffusion of a solute through a given solvent (Brodin, Steffansen and Nielsen, 2010). The diffusion coefficient depends on the molecular size of the solute molecule and the viscosity of the solvent (Brodin, Steffansen and Nielsen, 2010). Permeability coefficients are only for membrane calculation however, diffusion coefficients can apply to a free solution or a membrane and the higher the value of the diffusion coefficient the higher the value of the permeability coefficient (Sperelakis, 2011).

Einstein, assuming the solute radius is larger than the solvent radius, designed an equation for the diffusion of a solute through a solution; it was the theory of Brownian motion based on kinetic theory. The expression was derived for self-diffusivity (Manju Sharma, 2007). The diffusion coefficient depends on the molecular size of the solute molecule and the viscosity of the solvent (Brodin, Steffansen and Nielsen, 2010). The equation is the Stokes-Einstein equation:

$$D = \frac{RT}{N_A} \frac{1}{6\pi\eta r_u}$$

D = Diffusion coefficient (cm^2/sec)
R = Gas constant (J/molk)

T = Temperature (K)

N_A = Avogadro's number (mol)

Ɪ] = Viscosity (mPa.s)

r_u = Solute radius

The equation shows that the diffusion coefficient increases as viscosity and solute radius decrease. This could show a change in the diffusion coefficient value as the solute moves from the solution to a more viscous membrane: as the size of the drug decreases (smaller molecules), the diffusion coefficient increases, as it is easier for small molecules to diffuse through solutions and membranes. However, due to the smaller MW of the drugs, the MW may not be a large enough factor to cause large changes in the diffusion coefficient. The magnitude of the diffusion coefficient is a possible parameter to assist in the determination of the capability of a drug to undergo post-mortem redistribution. However, factors including the lipophilicity and the drug matrix could affect the expected outcome of the diffusion coefficient of a drug, showing the drug matrix could hinder diffusion (Larhed *et al.*, 1997).

The permeability of drugs can be determined from a flux experiment involving a specified concentration gradient and the resulting values can be compared with other values obtained at different concentration gradients. Then these values can be compared with similar experiments and the drug could then be categorised by permeability. Certain experimental conditions should be taken into account to produce a valid permeability value. These include keeping the concentration gradient constant or as constant as possible with a change no more than 10% throughout the experiment. The only concentration gradient present should be the gradient across the membrane and the diffusion of the drug is only via passive diffusion (Brodin, Steffansen and Nielsen, 2010).

An experimental study was carried out involving three mediums to compare the diffusion coefficients of a number of drugs, these included phosphate buffer, native pig intestinal mucus and purified pig gastric mucin. There were two main parameters that were compared, the charge of the drug and lipophilicity (log P). The charge had little effect on the diffusion coefficient however; the lipophilicity had a large impact, as the diffusion coefficient decreased as the lipophilicity increased in native pig intestinal mucus. However, there was no correlation between the two parameters in the phosphate buffer and the purified pig gastric mucin. Two similar drugs at a larger molecular size had reduced diffusion coefficients in the pig intestinal mucus. The more lipophilic drugs e.g propranolol had lower diffusion coefficients than the hydrophilic drugs e.g metoprolol. The factor producing the largest effect on the diffusion coefficients is the lipophilicity, for example a difference of log P 1.8 reduces the diffusion coefficient by half (Larhed *et al.*, 1997).

A further study compared two diffusion coefficients for morphine, determined from molecular weight, injected into the brainstem of cats, in relation to the distance the drug could travel in a certain period and the peak concentrations that could be reached. The two diffusion coefficients were 3×10^{-6} and 5×10^{-6} cm^2s^{-1}, and the length of the experiments were up to one hour. There was a comparison of five distances with 2 mm the furthest. The comparison showed the larger diffusion coefficient resulted in quicker detection at the furthest

distance as the drug diffused faster through the tissue. Post-mortem, ante-mortem and theoretical data were compared with similar results (Clark, Edeson and Ryall, 1983). This shows that the diffusion coefficient can be a useful indication of PMR that can be theoretically modelled.

2.2.1.3 pH

Glycolysis and the breakdown of creatine phosphate (CP) ante-mortem are necessary for the production of ATP and ADP. Immediately after death ATP concentration is high and stays constant for a short time as it is broken down and resynthesized as long as CP is present (Bate-Smith, 1956). This allows for certain processes within the body that require ATP, some involving control of the membrane gradients, to continue post-mortem. However, these processes eventually stop, as they require oxygen to produce the ATP. After death the oxygen levels deplete until eliminated over a short period of time and this results in the cessation of the ATP production (Butzbach, 2010). This occurs as the dephosphorylation process takes over the resynthesis of ATP and the concentration of ATP decreases until it is eliminated, the enzyme involved in the dephosphorylation process had not been identified however, the optimal pH range is pH 7.2-6.0. In addition, an acidic molecule (inosine monophosphate, IMP) and a basic molecule (ammonia) are also produced at the same rate as the ATP (Bate-Smith, 1956). This results in the anaerobic respiration of cells to occur converting ATP to ADP that leads to the production of lactic acid (Goff, 2009) and the reduction of the selectivity of the membranes allows the release of intracellular hydrolytic enzymes (Butzbach, 2010). As a result of the production of the lactic acid the cells become acidic reducing the intracellular pH, which denatures enzymes and eventually the plasma membranes leading to the release of the intracellular substances (Butzbach, 2010). This decrease in pH within the cells would alter the ionisation state of any drugs present especially basic drugs, which would become ionised in the acidic environment. This ionisation would allow the drugs to move around the body, as ionised drugs are less likely to diffuse through biological membranes. In addition, the increase of carbon dioxide (Vass *et al.*, 2002) and lactic acid (Donaldson and Lamont, 2013) in the blood would also decrease the pH of the body after death.

The pH of blood post-mortem has been shown to decrease to pH 5.1 within the first 20 hrs after death for humans (Sawyer, 1988). The intracellular pH has been shown to drop, from pH 7.20 to pH 5.70 over approximately 12 hrs after death (Bate-Smith, 1956). In addition, after sampling, the blood pH can still decrease if there is not inhibitor present including 0.06% fluoride (Straumfjord and Butler, 1957). This change in pH would not affect basic drugs if the value of the pH was still above the pKa, however if the pH falls below the pKa of the drug it would become ionised and it would become trapped in the current environment i.e. tissue cells (Macintyre and Cutler, 1988; Daniel, Bickel and Honegger, 1995; Daniel and Wöjcikowski, 1997) as ionised drugs cannot pass through membranes, it may only be able to travel via aqueous solutions including the bloodstream. However, the cell membranes break down after a period of time and the drugs would be released into the extracellular space in the acidic solution from the cell and due to this is able to be more easily redistributed (Cotran, Robbins, 1994). The length of time it takes for the breakdown of the cells depends on the site as cell death in the myocardium is observed approximately 40 minutes after death whereas the liver is approximately 1 to 2 hrs post-mortem (Cotran, Robbins, 1994).

Drugs that are present in the tissue cells at the time of death would need to be liberated from their binding sites (Creel, Lovich and Edelman, 2000) to be released into the surrounding environment. Previous study involving determining the stability of the $GABA_A$ receptors in the brain post-mortem, which binds to a number of anticonvulsant drugs. The results show that binding sites within the rat brain were reduced by 49% at 20 hrs post-mortem and in humans longer PMI was possibly the reason for increased loss of affinity for the receptor after death (Atack, Ohashi and McKernan, 2007). Another study demonstrated the rapid increase of morphine in rats within minutes after death and concluded it was a combination of the hydrolysis of morphine glucuronides to free morphine and also the release of morphine due to the reduction of pH which occurred within 2 minutes after death (Sawyer and Forney, 1988). After the release of the drug this would result in the drug diffusing into the nearest organ or vessel (Creel, Lovich and Edelman, 2000), which would increase the drugs' ability to be able to diffuse.

The reduction in the cellular pH results in rigor mortis by the locking of chemical bridges, which forms between 2-6 hrs after death and further develops over the subsequent 12 hrs. Rigor mortis usually begins when the pH of the body has been reduced to approximately pH 6.2 (Bate-Smith and Bendall, 1947). The onset of rigor mortis could be accelerated if the individual was exposed to intense exercise immediately prior to death as there is a loss of ATP (Bate-Smith, 1956) and an increased amount of lactic acid build-up in the body that would lower the cellular pH at an increased rate (Goff, 2009). As a result, the tissue cells would breakdown at a faster rate releasing the drug into the extracellular space. Movement of the drug would then begin at an earlier time than expected, with a possible result of the drug diffusing further around the body.

The amount of ATP has been found to be an important initial factor in the initiation of the autolysis and putrefaction processes and this depends on the amount of glycogen and CP initially present in the cells. The concentrations of these two substances (CP and glycogen) ante-mortem depend on the amount of food ingested, level of exercise prior to death and the how death occurs (Bate-Smith, 1956).

In relation to post-mortem values the expected pH at the commencement of rigor mortis, which is within 24 hrs after death, would be pH 6.2. This value is directly between the two analysed pH values of pH 5 and 7 for both the basic and the acidic drug (Borzelleca and Lowenthal, 1967), with pH 5 showing the slowest diffusion of the basic drug and pH 7 the slowest diffusion of the acid drug. At pH, 6.2 there would not be a significant reduction of diffusion for either type of drug.

2.2.1.4 Temperature

When an individual dies the active processes cease, which means that heat production is stopped leading to cooling of the body (Leinbach, 2011). The cooling of the body slows the decomposition process and as a result, this could slow down the diffusion of drugs post-mortem. The body has been shown to cool to ambient temperature within the first 18-20 hrs post-mortem (Fisher, 2003). The initial concept by Rainy was based on Newton's law of cooling, which states that cooling rate of a body is not linear but a declining value over time and is affected by numerous factors including heat conductivity, size, clothing and air movement/temperature (Kaliszan, Hauser and Kernbach-Wighton, 2009). In addition, there can be heat production post-mortem

(Henßge, 2004). Exercise before death can result in an elevated temperature for the first hour post-mortem (Henckel *et al.*, 2000), which could give an initial boost to the diffusion process. In addition, lower pH was observed immediately after death up to 6 hrs (Henckel *et al.*, 2000). This results in higher temperatures and lower pH that could ionise drugs and degrade cell membranes. Therefore increasing diffusion allowing the drugs to move further around the body after death. Also, the temperature increase could be attributed to the increase in anaerobic respiration after death (Hutchins, 1985).

A factor that could affect the rate of pH change would be temperature as the increase in temperature would increase the onset of autolysis and as a result increase the rate of putrefaction of the body (Haglund and Sorg, 1996) via the increase in bacterial growth (Goff, 2009). Another experiment had similar results showing a pH range of 6.9-5.6 over six hrs post-mortem, with the temperature of the pigs being forcibly reduced to reduce the pH fall. Immediately after death, the maximum temperature was 40°C and the subsequent temperature rose by 0.3 - 1°C and the pH fell by pH 0.2 - 0.3. The pH was seen to fall slower in the cooled carcasses, as the pH was pH 0.1 - 0.2 units higher throughout the experiment. The content of the glycogen decreased and the lactate increased as the temperature increased. The rate of the pH decrease was independent of the temperature above 37°C and dependant on the temperature when it fell below 37°C. The rate of decrease was < previous assumptions, which were 0.6 units per hour (0.01 pH units per minute), this experiment showed 0.29 pH units per hour. This experiment disagreed with the previous study concluding the rate of the pH fall is due to the temperature of the muscles and not the glycogen content (Maribo *et al.*, 1998). This could result in falsely elevated concentrations of the drugs in the sampling sites. In addition, tissues are hydrolysed and the acceleration of the process breaks down protein structures resulting in an increased level of basic nitrogen products including amines. These products eventually neutralise the acid build-up in the body increasing the environment to alkaline pH, which is favourable for bacterial growth and therefore increased level of putrefaction. The temperature for this process to occur needs to be within 21 – 38 °C, which is optimum for bacterial growth and this can be achieved both with deaths indoors and outside. Post-mortem temperature could be raised at death indoors by central heating, electric blankets and the physical condition of the individual including health and consumed medications. In relation to outdoor deaths, warmer climates or heat waves, submersion in warm water and hot cars (Zhou and Byard, 2011) could contribute to higher body temperatures that could increase the decomposition process.

2.2.1.5 Post-mortem blood movement

In addition to the environmental conditions that could affect the movement of drugs including temperature and pH, the physical process of drug movement is also possible after death. There have been changes in post-mortem drug concentrations recorded from a few minutes (Maskell, 2016) to a few hrs after death (Vorpahl and Coe, 1978; Pounder, Anderson and Watmough, 1994; Logan and Smirnow, 1996; Moriya and Hashimoto, 1999; Flanagan, Amin and Seinen, 2003). The contraction of heart muscles during the rigor mortis process have been shown to produce blood movement within the first 24 hrs post-mortem. This has been shown with contrast media as the compounds are forced through natural anatomical routes (Zapata, Luna, 1989). Therefore, the increase in the concentration of the small, basic drugs within this period could be attributed with this process of diffusion through vessels. In addition, another possible route is diffusion through a

concentration gradient as after death the pH decreases releasing the drugs from the cells initiating the diffusion (Zapata, Luna, 1989; Moriya and Hashimoto, 1999).

2.2.1.5.1 Post-mortem blood movement within 24 hrs after death

An animal study injected a contrast medium into the left ventricle of the heart that had been initially emptied of blood. Movement to central vessels was observed within one hour post-mortem and the contrast medium was visible in more peripheral sites including the renal arterial system within three hrs (Zapata, Luna, 1989). This was mainly due to the contraction of the heart muscles forcing the blood through the vessels, as there is a limited amount of putrefactive gases present at this point in the decomposition process. The temperature and pH would initially be at physiological conditions, 37°C and pH 7.4, and then within the first 20 hrs after death both would decrease to ambient temperature (Fisher, 2003) and a lower pH of approximately pH 5 (Sawyer, 1988). The changing conditions would allow the release of drugs from within the cells after death. The drugs would, depending on suitable conditions, be able to diffuse into surrounding organs and blood vessels, the blood vessels would carry the drug–laden blood around the body until the ionisation state of the drug changes due to change in pH and then diffuse into neighbouring organs. This would mean the dug concentrations in the original organs would decrease and as the drugs move around the body diffusing into other organs these concentrations would increase. These changes would affect the drug concentrations in different sampling sites that would be used at autopsy. In relation to amitriptyline, after death the drug is released from the lungs and diffuses into the heart and cardiac blood (Hilberg, Mørland and Bjørneboe, 1994). This could demonstrate that rigor mortis forces drug-containing blood to enter the heart increasing drug concentrations within the first 24 hrs after death.

2.2.1.5.2 Post-mortem blood movement later than 24 hrs after death

In the later stages of autolysis, up to 48 hrs post-mortem (Dent, Forbes and Stuart, 2004), the combination of decomposition processes including putrefaction increases the internal temperature, commonly above 50°C, inside the body producing an environment independent to the surroundings (Goff, 2009). This is favourable for the hydrolysis of the tissues that lead to the production of the basic amines that raise the pH resulting in further bacterial growth that advances the putrefaction process (Haglund and Sorg, 1996; Goff, 2009; Zhou and Byard, 2011). The gases produced by putrefaction cause further movement of blood after death. Both animal and human studies show that blood is able to move post-mortem (Fallani, 1961; Zapata, Luna, 1989). One of the possible mechanisms for the blood movement is a combination of the cessation of rigor mortis, fluidisation of the blood and the pressure increase due to the putrefaction gas that forces blood through the vessels (Fallani, 1961). At this stage of decomposition, the cells would have broken down and released any drugs present into the surrounding environment. As a result, the drugs would be transported in the blood initiating post-mortem redistribution. Other possible mechanisms for post-mortem blood movement are any physical movement of the body post-mortem, which includes transport to the mortuary. As changing the body position, especially before lividity was fixed, could transport drugs around the body to accumulate at different sampling sites (Fallani, 1961; Anderson and Jones, 1990). In addition, another possible factor that would need to be taken into account would be if there was any attempt to resuscitate the individual including

cardiopulmonary resuscitation (CPR) as the compressions would force the blood around the body falsely altering the drug concentrations at other sampling sites via redistribution (Yonemitsu, 1991).

Blood movement related to putrefaction gas has been shown that it does not begin until the rigor mortis process has ceased, which could occur between 24-84 hrs post-mortem (Goff, 2009), and the blood becomes fluid in the heart (Fallani, 1961). Initially, the blood moves around the heart into the pulmonary vessels and the aorta, which is possible due to the vessels having no resistance from the cessation of rigor mortis. As previously stated, at this stage the release of drugs have occurred from the tissue cells during autolysis, and possibly began to diffuse into the bloodstream (dependant on the physiochemical properties). As this is the first area of main blood movement there could be large changes in drug concentration from blood entering the heart via the surrounding vessels and the lungs (Hilberg, Mørland and Bjørneboe, 1994). As the amount of gas increases, this raises the pressure in the abdomen and the peripheral vessels become relaxed producing a blood flow into the peripheral sites. This occurred at the later stages of a human study carried out over 72 hrs (Fallani, 1961). This later stage empties the blood from the heart, which would transport drugs in the bloodstream to the more peripheral sites. This coincides with studies involving case reports showing a number of drugs, sampled at the femoral vein, can change concentration after death with an average time between admission to the mortuary and autopsy of 64 hrs. The results showed 20 from 52 drugs increased with a range of percentages with the lowest at 20% and the highest at 300% (Gerostamoulos *et al.*, 2012). In addition, fentanyl has also shown to undergo PMR in the femoral vein within the first 48 hrs after death with an increase of 5.6 fold between plasma concentration, taken 48 hrs after the fentanyl patch was applied and before sacrifice, and samples taken 48 hrs post-mortem (Zwart, 2012). This shows putrefactive gases do affect the movement of blood flow around the body however, the exact mechanism is still unclear, and the extent to which the production of the gas is affected by environmental conditions. As a result, it is still unclear how much the gases affect the movement of the blood around the body and therefore the significance of this factor in the post-mortem redistribution process are still unclear.

Previous work has shown that the length of time a cadaver has been buried affects the amount of mould present that could affect the blood flow. It is possible to add a fourth phase after 72 hrs post-mortem as the body loses fluids from the skin and body orifices due to the high abdominal pressure. This phase could include the movement of blood to the peripheral vessels and loss of fluid through the skin and other body orifices (Fallani, 1961).This could increase the drug concentrations in the femoral vein due to central blood being pushed into peripheral areas within the body.

However, there is an inconsistency between the rabbit and human study involving blood movement, which highlights contradictory evidence for the movement of blood within the first 40 hrs after death (Fallani, 1961). The contrast medium was introduced into the heart chamber, left ventricle in the rabbit study (Zapata, Luna, 1989), in both sets of cadavers. However, in the rabbit study the aorta was clamped and the chamber was emptied of blood before the medium was introduced, whereas in the human study the medium was introduced into the chambers with clotted blood still present (Fallani, 1961; Zapata, Luna, 1989). The clotted blood could have hindered the movement of the contrast fluid until the blood became fluid at a later stage. In addition, the

cadavers used in the human study had a range of post-mortem intervals between 7-72 hrs and as a result would have been in different stages of decomposition. In comparison, the rabbits were killed just prior to the experiment, and therefore represent a more realistic timeline of the decomposition process after death. Further work would need to be carried out on the post-mortem circulation studies due to the contradictory result between the two experiments.

Drugs move along blood vessels via diffusive and convective effects, which could be slowed down, particularly with hydrophobic drugs by the binding and subsequent release of the drug to fixed hydrophobic sites in the arterial wall (Benet *et al.*, 1996). This has been shown to slow the hydrophobic drugs down significantly enough for larger hydrophilic drug molecules to distribute though the vessels faster (Creel, Lovich and Edelman, 2000). A study carried out using paclitaxel, a hydrophobic compound, and calf carotid arteries was carried out to determine the concentration at each layer within the vessel at equilibration (72 hrs) for a number of different concentrations of the drug. In addition, a simulated pressure gradient through the intact arteries allowed for the determination of the drug distribution after it was introduced into the lumen of the artery and around the outside of the artery. The results showed that the drug equilibrated within the 72 hrs and the concentration had no effect on the partitioning of the drug with the tissue, which was significant as the tissue concentration was greater than the solution concentration. An experiment was carried out at equilibrium to see how much of the drug partitions into the artery, the results showed that the drug concentrations in all sections of the tissue exceeded the surrounding solution concentration showing the drug highly partitions into the tissue (Creel, Lovich and Edelman, 2000). Drug partitioning could affect the resulting drug concentrations as if there was a high affinity of the drug for the tissue this would reduce the amount of available drug in the vessel for analysis, which could lead to an underestimation of the drug concentration. In relation to PMR, the blood samples would show lower drug concentrations than tissue samples and the longer the post mortem interval the higher concentration of drug would partition in the tissue.

2.2.1.6 The inconsistent method of estimating ante-mortem drug concentrations from post-mortem samples

Another factor that could determine the extent of PMR a drug could undergo is the central to peripheral ratio, which is the comparison of drug concentrations from central sites mainly the heart to peripheral sampling sites including the femoral vein. One of the factors that affect the central to peripheral ratio of a drug is the period between death and removal of the sample, as the longer the interval the more time allowed for redistribution. This would result in drug movement between the sampling sites and therefore the ratio could be affected, which shows the ratio alone cannot be used to determine the extent of PMR for a drug. It was suggested to estimate the ante-mortem concentration of drugs using the post-mortem drug concentrations taken and analysed from the autopsy. Between the six cases there were seven drugs tested that included propranolol, amitriptyline, dothiepin, dextropropoxyphene, paracetamol, salicylate, and methadone. The central to peripheral ratios were sourced for each of the drugs. This was compared with the ante-mortem to post-mortem ratio to see if there is any correlation between the capability of the drug to redistribute and the difference in the drug concentration between death and the time of sampling. The results showed from the six cases that there was a correlation between the two parameters; the drugs with a high central to peripheral

ratio (C/P) also had a high post-mortem to ante-mortem ratio (PM/AM). Propranolol was the only exception with a lower PM/AM than the C/P, which could have been due to the time interval between the ante-mortem sampling and death being unknown. This could show that the interval between ante-mortem sampling and death is an important factor that would affect the PM/AM ratio, which would affect the redistribution conclusions about the drug. The post-mortem concentrations of the drugs were equal or higher than the ante-mortem concentration, which shows that is not advisable to estimate ante-mortem drug concentrations from the post-mortem concentrations. Also, due to the fact that the difference in the concentrations could not be explained by a general rule as the differences varied between each drug (Cook, Braithwaite and Hale, 2000).

In another case, specifically involving fentanyl, this showed this drug undergoes minimal PMR over 12.6 hrs (McIntyre and Gary, 2014) however, in the later stages of PMI there was PMR present for the same drug with a 5.6-fold increase (Zwart, 2012). In addition, this is further confirmed by the extensive work carried out relating the post-mortem mean to the therapeutic range of 129 drugs, showing a variable range in responses that were drug dependant. Approximately half of the drugs had directly compared to the therapeutic values, showing minimal post-mortem change. The other drugs had variable values for the post-mortem to therapeutic ratio, showing some drugs underwent PMR and some drugs reduced after death (Launiainen and Ojanper, 2014).

2.2.1.7 Effect of PMR on certain organs

PMR can affect some sites within the body more than others, the solid organs that show the highest concentrations of drugs are the lungs (Pounder and Adams, 1996), which is mainly due to the high density of capillaries (Anderson and Jones, 1990) and the liver (Pounder and Adams, 1996). Animal studies have shown the release of a significant drug concentration in the lungs could diffuse through the vessels or through a concentration gradient to increase the concentration of cardiac blood (Hilberg, Mørland and Bjørneboe, 1994; Moriya and Hashimoto, 1999). Also, the stomach is a source of diffusion of drugs (Pounder, Fuke and Cox, 1996) and aspiration of stomach contents could correlate with the increase in heart blood concentrations (Hilberg *et al.*, 1992). These processes could cause post-mortem diffusion of drugs via the natural anatomical route into the heart and related vessels (Anderson and Jones, 1990) including the pulmonary artery and vein and the inferior vena cava. Furthermore, the diffusion could progress into the cardiac chambers resulting in falsely elevated concentrations of the drug (Anderson and Jones, 1990). However, there is less redistribution in the peripheral vessels as there are limited sites for redistribution to take place, with only muscle and fat for drugs to redistribute from to the vessel (Cook, Braithwaite and Hale, 2000). As a result of these conclusions it has been stated in many papers that the most appropriate site for sample collection is the femoral vein as it is in a peripheral region where there is less redistribution (Anderson and Jones, 1990; Prouty and Anderson, 1990; Yarema and Becker, 2005).

2.2.1.8 Post-mortem blood drug concentration changes in the femoral vein

There are more drug depots in the viscera; as a result, there would be more movement of the drugs between the vessels and organs in this region of the body. However, there is less redistribution in the peripheral vessels as there are limited sites for redistribution to take place. The location of the femoral vein is further

away from the organs and is only surrounded by muscle and fat, which limits the location from which the drugs could be transferred into this vessel (Cook, Braithwaite and Hale, 2000). This classes blood in the femoral vein as a peripheral sample due to the location of the vein away from the main organs within the body (Yarema and Becker, 2005). As a result, the concentrations in the peripheral samples would take longer to increase allowing for a more realistic value of the concentration of the drug at the time of death over a longer post-mortem period. As a result of these conclusions it has been stated in many papers that the most appropriate site for sample collection is the femoral vein as it is in a peripheral region where there is less drug redistribution (Anderson and Jones, 1990; Prouty and Anderson, 1990; Yarema and Becker, 2005; Zilg, Thelander and Giebe, 2017). The vein would be chosen over the artery as there are higher drug concentrations when comparing the two vessels with a ratio of 1.2 for morphine between the femoral artery/vein (Cameron, 2006)**.** The ideal way of sampling blood from the femoral vein has been shown to cross clamp or ligate the vessel then use a needle to extract the blood sample (Anderson and Jones, 1990). However, there has been a study that investigated four drug classes comparing blind stick sampling of the vessel to clamped sampling with a good correlation showing no difference between the sampling methods (Hargrove and McCutcheon, 2008).

There are possible drug depots that could redistribute into the femoral vein, which includes the skeletal muscle, body fat and the bladder (Moriya and Hashimoto, 2001; Kennedy, 2010). A study was published investigating the homogeneity of leg skeletal muscle, as it could be used as an alternative matrix when conventional samples are unobtainable. The results showed uneven distribution of drugs within skeletal muscle, which was determined for nine drugs, this shows that there is accumulation in the muscle which could redistribute to the femoral vein after death (Pounder, 1997). In addition, there have been post-mortem cases showing both higher concentrations of drugs in the femoral vein than the cardiac samples (Pounder, 1993) and an increase in a drug concentration between ante-mortem sampling and autopsy (Cook, Braithwaite and Hale, 2000). Another case involved observing an increase of 100% in the drug concentration between the ante-mortem sample and the sample taken at autopsy (McIntyre and Gary, 2014). Also, higher concentrations were found in blood taken from the same site of 149 cases with an average of 64 hrs between the two samples (Gerostamoulos *et al.*, 2012). As a result of these findings it can be shown that the drug concentrations determined from femoral samples taken at autopsy cannot be taken as the concentration of the drug present at the time of death (Anderson and Jones, 1990).

Previous papers have stated that drugs, including methamfetamine, redistribute faster in pulmonary veins, resulting in earlier post-mortem drug concentration increase (Pélissier-Alicot *et al.*, 2003), than in arteries due to the difference in the thickness of the vessels. Specifically relating to the lungs, methamfetamine has been suggested to diffuse through the pulmonary veins into the left cardiac chamber thus increasing drug concentrations further than in the right chamber (Moriya and Hashimoto, 2000), however no experiments have been carried out to prove this. A direct comparison between matching pairs of arteries and veins show there is higher drug concentrations in the arteries and these concentrations increase with PMI (Chiou, 1989). The results are dependent on the absorption, distribution, metabolism and excretion (ADME) phase, during the absorption and distribution phase the results stand with higher drug concentration in the arteries. However, if

the drugs were in the elimination phase at the time of death there would be higher drug concentrations in the veins in comparison with the arteries (Chiou, 1989). In relation to this research, the femoral vein is used for autopsy sampling due to less redistribution. However, if there is drug diffusion from the bladder and the vein is more susceptible to drug alteration due to the thinner walls then this site could be affected. In addition, this area of research has limited experiments on how the bladder behaves after death: how it breaks down if drugs diffuse out of it and if so how much over what time period. The gap in the current research is the lack of data on the behaviour of the bladder after death and how this affects the diffusion of drugs out of the bladder and in turn the possible alteration of femoral vein samples.

There have been investigations into a number of organs in regards to PMR; however, there is limited research on the bladder. This issue was raised with a case report published in Japan involving an individual that had been found after nine days with higher concentration of diphenhydramine and dihydrocodeine in the femoral vein compared to the cardiac blood (Moriya and Hashimoto, 2001). This was associated with the breakdown of the bladder releasing drugs into the surrounding areas, which then diffused into the femoral vein increasing the concentration of the drugs. The higher femoral to cardiac blood is significant as there is usually greater redistribution of drugs around the central vessels due to higher concentrations of drugs in certain organs.

PART 3 – Permeability of the Bladder

3.1 The Bladder

The bladder is a muscular, hollow organ that is secured in place by the peritoneum, collapsed when it is empty and spherical when it contains urine. The bladder is a storage organ, which then removes urine from the bladder via the urethra in response to nerve impulses. This action is voiding of the bladder or urinating. The bladder can contain up to 800ml however, the impulse to void the bladder arises when the bladder contains upwards of 200-400ml (Tortora, 1995).

The bladder is located in the pelvis, above and behind the pubic bone (See Figure 1.1). The bladder wall consists of four main layers (See Figure 1.2) that include the mucosa (sectioned into the transitional epithelium and the lamina propria), sub-mucosa, detrusor muscle, and the adventia. The mucosa is the innermost layer of the bladder wall and is separated into the transitional epithelium and the lamina propria. The transitional epithelium is also known as the urothelium. This has been shown to be the layer that controls the permeability of the bladder, which causes the bladder to be an impenetrable barrier to most substances (Khandelwal, Abraham and Apodaca, 2009; GuhaSarkar and Banerjee, 2010). Also, it prevents the contents from transferring into the bloodstream (Acharya *et al.*, 2004). The urothelium has several layers including the hydrophilic lycosaminoglycans (GAG) mucin layer, plaques, tight junctions, umbrella cells, intermediate cells, and the basal cells, in order from the lumen to the sub-mucosa. The basal cells are the smallest set of cells with a 5-10 µm diameter, the intermediate cells are approximately 20 µm, and the larger cells are the hexagonal umbrella cells between 50-120 µm. The regeneration of cells is a constant process that occurs by the fusion of the cells in each layer to form the layer above, for example the basal cells fusing to form the intermediate layer.

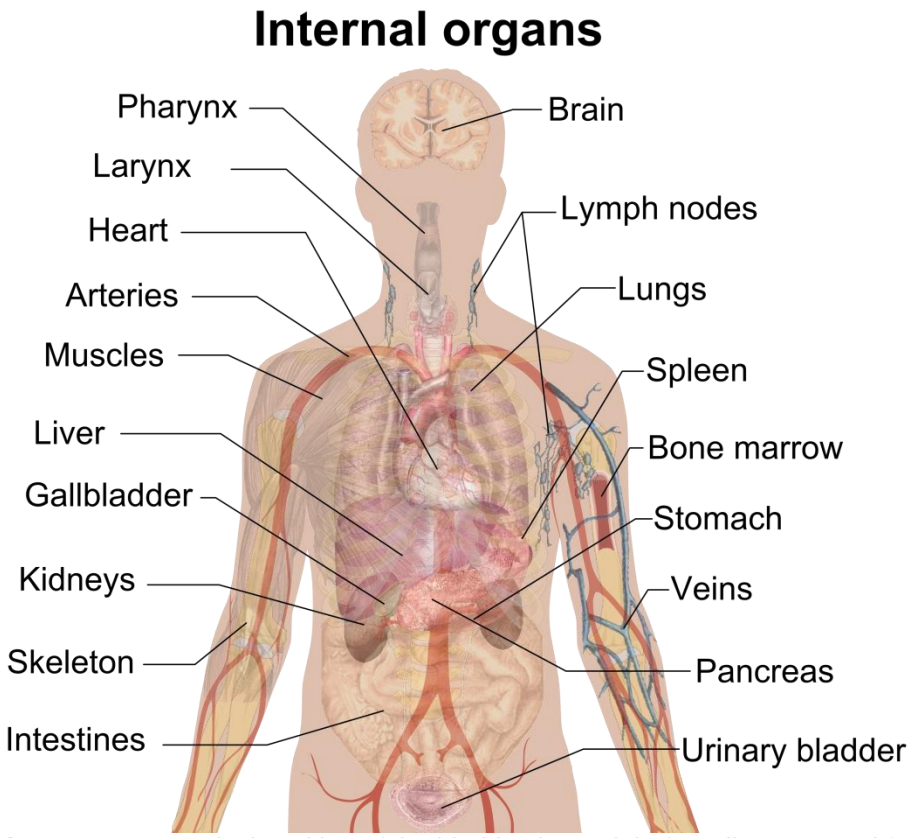

Figure 1.1 Anatomical position of the bladder (Copyright free diagram used from: https://commons.wikimedia.org/wiki/File:Man_shadow_anatomy.png)

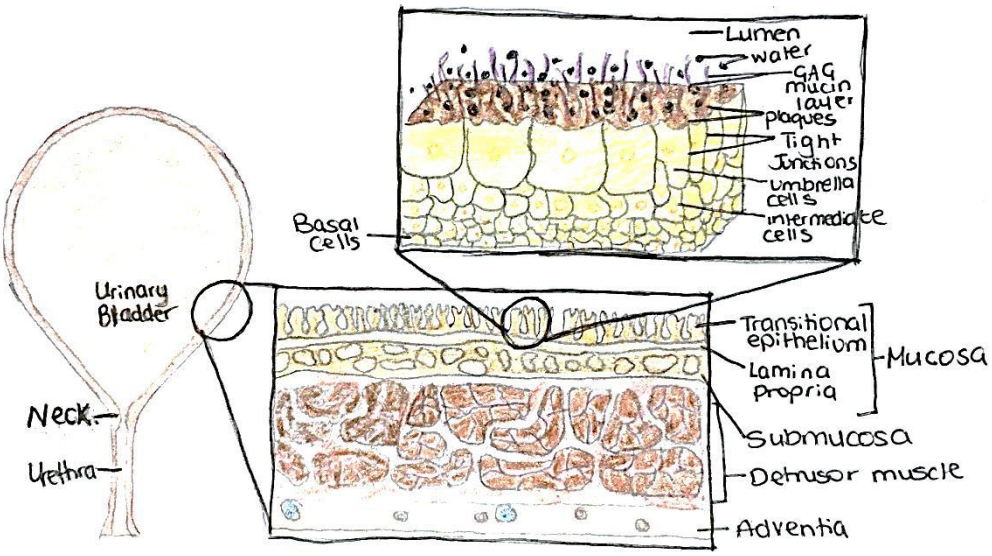

Figure 1.2 Layers of the bladder (Diagram based on (GuhaSarkar and Banerjee, 2010) and generated by the author)

The umbrella cells are the main cause of the impenetrable nature of the bladder membrane; this is mainly due to tightly packed hexagonal plaques covering most of the apical membrane increasing the thickness of the outer leaflet of the membrane (GuhaSarkar and Banerjee, 2010). This structure is comparable with 18 mammalian species including human bladders. Specifically including rat, calf, pig and human the similarities have been extended to a microscopic level with the individual units that produce the structure of the membrane, (Warren, 1973). This demonstrates that certain mammalian bladders can be used to mimic human bladders in research. Also, the presence of hydrophilic glycosaminoglycans (GAGs), which includes proteoglycans and glycoproteins (Hurst, Roy and Min, 1996), forming a layer on top of the umbrella cells in the lumen of the bladder inhibits the diffusion of substances out of the bladder. This layer prevents the adherence of solutes (Poggi, Johnstone and Conner, 2000) to the bladder membrane and is known as the bladder protector factor (Soler *et al.*, 2008). The permeability of the membrane increases when the GAG mucin layer and the umbrella cells undergo degradation (GuhaSarkar and Banerjee, 2010). This is a possibility as a previous study has uncovered that the bladder membrane quickly breaks down after death with the loss of the umbrella cells, which are responsible for maintaining the impermeable barrier (Jost, Gosling and Dixon, 1989). The lower layers would then be exposed (Newman, 1981) and would allow substances, including drugs, to pass through the bladder into the surrounding environment possibly resulting in post-mortem redistribution.

There are limited studies on the bladder as it has shown that it degrades quickly after death resulting in a limited number of post-mortem samples (Jost, Gosling and Dixon, 1989). However, this would be useful in terms of determining the length of time this semi-impermeable barrier remains viable after death. There are early signs of damage to the bladder urothelium when it undergoes 60 minutes or more of ischemia, resulting in the disruption of the tight junctions between the superficial cells in the lumen of the bladder wall. This funnel-shaped urothelial damage descends into the lower regions of the bladder membrane penetrating the lamina propria (Koroäec and Jezernik, 2000).

Bladder related research has been focussed around cancer treatment and increasing permeability of a drug to the affected region of the bladder (Wientjes *et al.*, 1991). There is a limited amount of studies carried out on the degradation of the bladder and the affect this has on the permeability. The bladder has shown to be penetrable to drugs when damage occurs during life and studies have shown to be able to intentionally damage bladders to mimic conditions such as interstitial cystitis. The work included showed increase in the permeability of the bladder of six patients with interstitial cystitis that was initially tested with animals. The direct test compared intact bladders with damaged (50% acetone rinse) and distended bladders by analysing the blood after instillation of two sugars (4% lactulose and 1% rhamnose) into the bladder. Neither sugar was detected in the intact bladders, however both sugars were detected and quantified in the damaged and distended bladders (Erickson *et al.*, 2000). There have also been two other studies involving the intentional damage to bladders using ovalbumin (Lavelle *et al.*, 1998) and protamine sulfate (Lavelle *et al.*, 2002) to recreate the damage caused by interstitial cystitis. These studies were used to look at the regeneration of the bladder overtime after the damage has occurred. The second study involved reproducibly damaging the bladder membrane using a molecule known to specifically damage the umbrella cells of the bladder to

observe how the bladder repairs itself after damage. The results showed that after damaging the bladder with different concentrations of protamine sulfate (PS) the umbrella cells were targeted and damaged. The cells in the lower layers then adapted and formed new umbrella cells overtime and within 5 days the bladder returned to normal functionality (Lavelle *et al.*, 2002). In relation to the treatment of bladder cancer, the permeability of the porcine bladder to polymers used for intravesical insertion of drugs into superficial tumours in the bladder was tested. There was good correlation between a previous diffusion model and the results. The results show that using both polymers to open the tight junctions within the lumen of the bladder the diffusion of the hydrophilic drug was increased (Grabnar, Bogataj and Mrhar, 2003). This shows that with disruption to the bladder membrane drug diffusion is possible during life, which could also translate to the damage caused after death during the decomposition process that could result in similar increase in permeability.

Numerous drugs have been found to passively pass through the bladder wall (Borzelleca, 1965) including barbiturates, basic drugs (atropine, neostigmine and physotigmine) (Borzelleca, 1959), nicotine (Borzelleca, 1963) and phenobarbital, salicylic acid and quinine (Borzelleca and Lowenthal, 1967). This is increasingly important when involving drugs that remain unchanged when being excreted through the renal system as there would be storage of the parent drug that could leak into the peritoneal cavity (Tucker, 1981) increasing the ratio of the parent to metabolite ratio. A few drugs that have been reported to be excreted unchanged include anticonvulsant drugs gabapentin (Wong and Eldon, 1995) and pregabalin (Corrigan and Pool, 2001), the antineoplastic agent methotrexate (Huffman, 1973) and the antipsychotic and antiemetic levosulpride (Forgione, 1995). An in-vitro study was carried out looking at different parameters that could affect the movement of four drugs (nicotine, salicylic acid, quinine, and pentobarbital) across the bladder wall of rabbits. The parameters included pH, concentration, bladder volume, and temperature. The results showed that the lower the pH the slower the basic drug (nicotine) diffused out of the bladder and the opposite effect for the acidic drug (salicylic acid). The pH range for the basic drug included pH 5.3-9.0 and pH 3, 5, and 9 for the acidic drug, which extends the range that had been recorded for the post-mortem urine present in the bladder after death with a range between pH 4.6-8.5 (Cook, Strauss and Caplan, 2007). Changing the temperature from 37°C to 27°C, 32°C, or 42°C decreases the rate at which the drugs diffuse across the membrane. The higher the concentration, comparing 1, 10, and 100 mM, of the drug the higher the rate of diffusion, however the value reached a plateau at the highest concentration (100 mM). Comparison of different volumes of fluid in the bladder, 1, 3, 9 and 18ml solution/kg body weight, resulted in less drug (both concentration and rate constant) diffusing from the bladder at higher volumes (Borzelleca and Lowenthal, 1967).

One study looked into the permeability of four drugs with different physicochemical characteristics including molecular weight, pKa, solubility at pH 7.4, log $K_{o/w}$ and log D through porcine bladder. The result showed that alongside the differences in the experimental setup the hydrophilic drugs had a higher penetration into the bladder than hydrophobic ones. In addition, the mathematical model describing the diffusion of drugs through the bladder membrane concurred with the experimental results (Moch, Salmon and Armesto, 2014).

It has been shown that the organ blood flow to the bladder is reduced when the bladder is distended (Lapidus, 1974), which shows that drugs could not diffuse into the bloodstream and be transported to other sampling

sites via this method. The more likely is that bladder distention, which temporarily increases permeability, and damaged bladders could increase the permeability of the bladder. Urine retention in the bladder has been studied suggesting a link between drug use including medication and the drugs ability to interrupt the process involving fully emptying the bladder (Abrams, Cardozo, 2002). The process of emptying the bladder is a complicated process named micturition, which involve the nerves in the urethral sphincters that prevent the loss of urine until the response to neurotransmitters that are bound to receptors that can initiate or cease the process of micturition. Certain drugs have been linked to interfering with the process, especially in the elderly, that include benzodiazepines (diazepam and clonazepam), antipsychotics (chlorpromazine) and antiarrhythmic (disopyramide). There are two types of urinary retention: acute and chronic and the acute has had more research carried out. However, a chronic study was carried out showing the use of a number of drugs had resulted in chronic urinary retention including common cold medications, eperisone (antispasmodic) and amitriptyline (Kurasawa, Kotani and Kurasawa, 2005). In addition, a computed tomography (CT) based study resulted in a correlation with higher urine volume in the bladder, above 330 ml, with a positive toxicology result suggesting if an individual had a large amount of urine present this urinary retention could be due to the presence of drugs (Rohner and Franckenberg, 2013). These results show there could be an issue if there is a correlation between higher urine volume and the presence of drugs and in addition, the higher the urine volume the thinner the bladder walls. This could increase the diffusion of drugs from the bladder and have a higher probability that the bladder could be a source of PMR in relation to changes in the femoral vein drug concentrations. However, some of these studies were based around the living; less work has been carried out looking into the post-mortem breakdown of organs including the bladder. The studies that have been carried out are only looking into the change in drug concentration from different sites within the body over time (Prouty and Anderson, 1990)

3.2 Drugs

3.2.1 Model compounds to investigate PMR from the bladder

There have been studies that suggest a number of different dyes to test the permeability of the bladder urothelium, which include trypan blue (Monson *et al.*, 1991), indigocarmine (Monson *et al.*, 1991; Koroäec and Jezernik, 2000) and rhodamine B. The most suitable dye would be rhodamine B as it has been used previously as a contrast reagent for initial tests of the vascular tissue in the in-vitro study of post-mortem permeation of morphine and its metabolites through human veins (Skopp *et al.*, 1997). This substance would also give a visual indicator that could be easily identified on a UV-spectrometer. Rhodamine B (See Figure 1.3) is a lipophilic compound, that has been shown to passively diffuse into liver cells in previous work independent of temperature (Olinga *et al.*, 2001). The molecular weight is 479, which is a small molecule resulting in size not restricting the compound from diffusion through the tissue (Qi, Gao and Zhang, 2013). The basic pKa of this compound is reported as 4.2 (Zhang *et al.*, 2011) and the log P is 2.43 (Mah *et al.*, 2013).

In relation to the CT-based section of this research the suitable compound was identified as silver nitrate, which is an inorganic compound with a chemical structure of $AgNO_3$ that does not harm human cells (DeSanti, 2001). The molecular weight is 169.872 g/mol, which is a small molecule resulting in size not restricting the compound from diffusion through the tissue. The pH of aqueous silver nitrate is approximately pH 6 (Merck, 2001). This radio-opaque compound (Raj *et al.*, 2014) has been used in previous work to observe the amount of silver nitrate infusing into dental cavities (Carrera *et al.*, 2015) and also the penetration of this contrast agent into embryonic mice jaws to understand the developmental stages of the teeth (Raj *et al.*, 2014).

3.2.2 Drugs to investigate PMR from the bladder

Antidepressants are a widely known class of drugs that are prescribed for depression, anxiety disorders including panic disorder, somatic disorders including fibromyalgia and others including bulimia (Stahl, 2003), however they are also abused being the cause of death in suicides (Bynum *et al.*, 2005). There are different classes of antidepressant including the original tricyclics, serotonin reuptake inhibitors (SSRIs) and a mixed array of uptake inhibitors including monoamine oxidase inhibitors (Mehta, 2005). A common antidepressant is amitriptyline first prescribed in 1961 and metabolises into nortriptyline, which is the mono-N-desmethyl metabolite of the parent compound (Baselt, 2008) (See Figure 1.4 and 1.5). Both drugs are still present in numerous cases (Statistics, 2013). As a result, to keep the research current using drugs with known pharmacokinetic effects, amitriptyline and nortriptyline were chosen to be used in the bladder diffusion studies. A number of previous studies have been carried out using amitriptyline in relation to PMR from a number of biological tissues (Hilberg *et al.*, 1992, 1993; Hilberg, Mørland and Bjørneboe, 1994). As a result, due to the previous information on the drug and that is undergoes PMR amitriptyline would be a good drug to begin the research into this area of PMR from the bladder. Concentration changes of amitriptyline have been shown to occur within 2 hrs after death (Hilberg *et al.*, 1993). In addition, it was shown that the diffusion from the stomach, gastrointestinal tract and the lungs played a major part in post-mortem redistribution of the antidepressant drug amitriptyline (Hilberg *et al.*, 1992, 1993; Hilberg, Mørland and Bjørneboe, 1994).

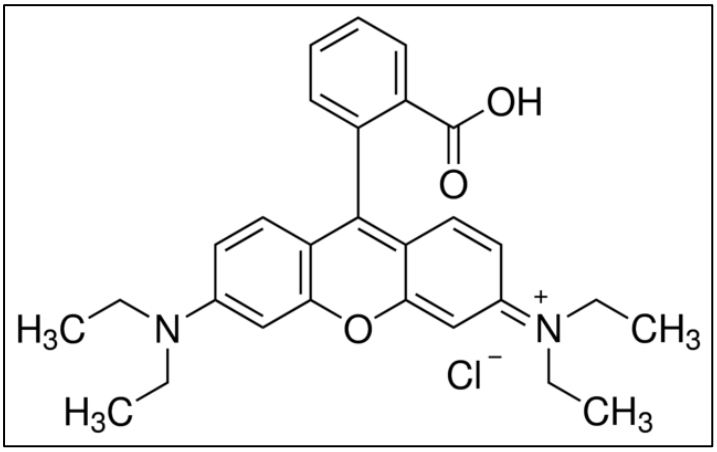

Figure 1.3 Image of the structure of rhodamine B

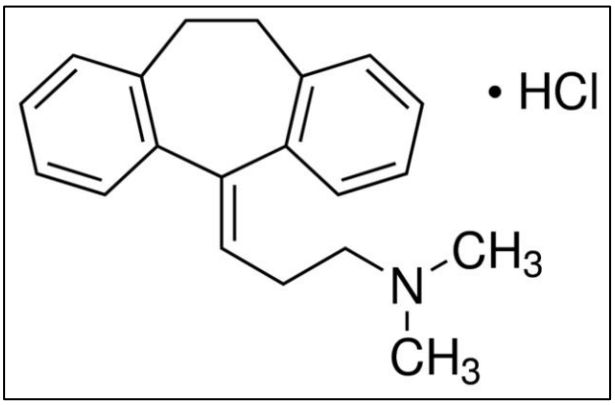

Figure 1.4 Structure of amitriptyline

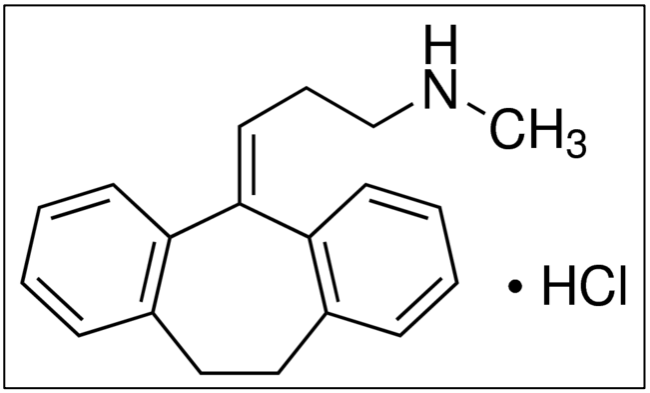

Figure 1.5 Structure of nortriptyline

3.4 Aims and Objectives

The primary aim of this study is to determine the role (if any) that the bladder may play in post-mortem redistribution (particularly from the bladder to the femoral vein). This study will utilise two model compounds rhodamine B, amitriptyline and the amitriptyline metabolite nortriptyline. In the CT study, silver nitrate will be utilised. The study will aim to look at the time scale of any diffusion from the bladder and the most suitable model to use to study diffusion of drugs from the bladder in the future.

3.4.1 Diffusion of drugs using Franz cells

The aim of this section of research is to determine if there is diffusion of drugs (rhodamine B, amitriptyline, and nortriptyline) from porcine bladder sections in-vitro. Other parameters included in these experiments were temperature, solution pH, tissue degradation, bladder volume and drug concentration.

This project is split into two sections involving the validation of drugs on the HPLC and UV and the bladder diffusion studies. The HPLC and UV were used for the validation of the chosen drugs according to the Scientific Working Group for Forensic Toxicology (SWGTOX), standard practices for method validation in forensic toxicology. Franz cells were chosen as there have been many previous studies using diffusion chambers to determine the diffusion coefficients of drugs. Rhodamine B would be used as the model compound and two antidepressants (amitriptyline and nortriptyline) used as commonly found in post-mortem cases. The length of the bladder diffusion experiments would be 5 days to mimic the method by Skopp (Skopp *et al.*, 1997) for the validation of the diffusion chambers, would be carried out over 5 days due to this being the length of the proposed experiments.

3.4.2 Diffusion of drugs through whole bladders

The aim of this section was to use intact porcine bladders to determine if solution pH, initial rhodamine B concentration, and bladder volume would affect the amount of drug concentration that would diffuse through the bladder tissue over 5 days.

The in-vitro nature of these experiments would prevent the bladder being in the natural state (inside the body) that would be found in deceased individuals. However, using whole bladders would increase the similarity to casework; similar parameters to the bladder section experiments described above would be included. In addition, two other parameters were included, variable initial volumes and concentrations. These parameters could determine if the volume of solution in the bladder and varied concentration could affect drug diffusion through an intact bladder after death. The length of the experiments was also carried out over 5 days for consistency.

3.4.3 In vivo study involving the diffusion from the bladder in a rat model.

As the two previous sections (3.4.1 and 3.4.2) used *in vitro* techniques it was important to investigate the diffusion in a whole animal and how this relates to the *in vitro* data and also the case data from (Moriya and

Hashimoto, 2001). The most suitable model was a rat that was scanned using a micro-CT scanner using silver nitrate as a contrast agent.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 General Chemicals and Reagents

The drugs used for this research included amitriptyline HCl (Sigma, UK) and nortriptyline HCl (Sigma, UK). The compounds used in the *in vivo* rat study were sodium diatrizoate hydrate (Sigma) and silver nitrate (ReAgent). The two buffers used phosphate buffer tablets (Sigma) and ammonium acetate (Sigma). The chemicals used to alter the pH of the buffer solutions were sodium hydroxide, ammonium hydroxide, acetic acid, and orthophosphoric acid, which were all of Analar grade. The HPLC solvents, methanol, and acetonitrile were HPLC Grade (Fisher). The buffer, triethylammonium phosphate solution (TEAP) (Sigma, 1M in H_2O) was used to stabilise the pH in the HPLC mobile phase. Rhodamine B (Sigma) was the chemical used as a model for the bladder diffusion studies. The disinfectant for the biological tissues was trigene (Trigene advanced).

2.1.2 Franz-Cell, Whole Bladder, and HPLC-DAD Solutions

20 mM Phosphate buffer (pH 7.4): 500 ml of 20 mM phosphate buffer solution (PBS) was prepared from five phosphate buffer tablets that had been crushed using a pestle and mortar and dissolved in deionised water. The solution was stirred on a magnetic stirrer for approximately 10 minutes to ensure a homogenous solution. A pH meter was used to record and adjust the pH to 7.4 using either orthophosphoric acid or dilute sodium hydroxide solutions. In order to degas the solution (to reduce the incidence of bubbles in the Franz cells) the solution was sonicated.

20 mM Ammonium Acetate Solution (pH 5): 500 ml of 20 mM ammonium acetate solution (AA) was prepared using 0.77 g of solid ammonium acetate weighed out using an analytical balance, then dissolved in deionised water. The solution was stirred on a magnetic stirrer for approximately 10 minutes to ensure a homogenous solution. A pH meter was used to record and adjust the pH to 5 using either ammonium hydroxide or acetic acid. In order to degas the solution (to reduce the incidence of bubbles in the Franz cells) the solution was sonicated.

2.1.3 Drug Stocks

Most solid drug standards are provided as a salt or hydrate, such as amitriptyline hydrochloride. The weight of the salt or hydrate must be corrected when preparing liquid drug standards. In order to have the correct concentration of liquid standard the following equation was used.

$$Volume\ of\ solvent\ required\ (ml) = \frac{\left(\frac{Weighed\ amount\ of\ drug\ standard\ (mg)}{(\frac{Formula\ weight\ of\ drug\ salt)}{Formula\ weight\ of\ drug\ base)}}\right)}{Concentration\ of\ standard\ required\ (\frac{mg}{ml})}$$

The following drug stock solutions were prepared: rhodamine B (100 and 200 mg/L), amitriptyline (1000 mg/L), nortriptyline (1000 mg/L).. All standards were prepared using deionised water, phosphate buffer pH 7.4, and ammonium acetate pH 5. The stock solutions were stored in 8 ml clear glass vials at 4°C until use.

2.1.4 Storage

The buffer solutions were prepared fresh at the start of each experiment, sealed with Parafilm and stored in the fridge at 4°C for the duration of the experiment. Two weeks was the maximum storage time for the drug solutions in buffer.

2.1.5 Calibration curve and Quality Control Sample Preparation

The calibration curve for the UV-Visible spectrometer comprised: 0.16, 0.31, 0.62, 1.25, 2.5, 5 and 10 mg/L. Quality control samples including high quality control (HQC) (5 mg/L) and low quality control LQC (0.5 mg/L) were used alongside the calibration standards to calculate the reproducibility of standard preparation using different stock solutions. The number of calibration standards were chosen in accordance with SWGTOX guidelines (SWGTOX, 2013).

2.1.5.1 Rhodamine B

A serial dilution was prepared for the calibration standards; 1 ml of the buffer relevant solution was added to the 10 ml volumetric flask to promote better mixing. Then 1 ml of the 100 mg/L rhodamine B calibration stock solution was added to the flask. The solution was made up to volume with the relevant solution (PBS or AA) producing a 10 mg/L calibration standard. Half of the top standard was removed to a fresh volumetric flask and made up to volume with the relevant solution. This was repeated until seven standards were obtained 0.16 mg/L – 10 mg/L. The two quality control standards were HQC (5 mg/L) and LQC (0.5 mg/L). The HQC was prepared by transferring 500 µl of the rhodamine B quality control (QC) 1000 mg/L stock solution to a 10 ml volumetric flask and made up to volume with the relevant solution. The flask was inverted several times to ensure a homogenous solution. The LQC was prepared by removing 1 ml of the HQC solution, then transferred to the LQC (0.5 mg/L) volumetric flask and made up to volume with the relevant solution.

2.1.5.2 Antidepressant Drugs

Preparation of the top calibration standard (10 mg/L), 100 µl of each 1000 mg/L analyte (amitriptyline and nortriptyline) calibration stock solution was added to a 10 ml volumetric flask. Initially, 1 ml of the relevant solution was added to the flask to promote better mixing. Then made up to volume with the relevant solution (PBS or AA).The calibration was prepared as a serial dilution therefore, 5 ml of the 10 mg/L was transferred to a clean 10 ml volumetric flask and made up to volume with the relevant solution. This was repeated until seven standards were obtained 0.16, 0.31, 0.62, 1.25, 2.5, 5, and 10 mg/L. All solutions were inverted to mix the solutions.

The two quality control standards were HQC (5 mg/L) and LQC (0.5 mg/L). To prepare the HQC, 50 µl of each 1000 mg/L analyte (amitriptyline and nortriptyline) QC stock solution was added to a 10 ml volumetric flask and made up to volume with the relevant solution. The volumetric flask was inverted several times to ensure a homogenous solution. To prepare the LQC, 1 ml of the HQC solution was transferred to the LQC (0.5 mg/L) volumetric flask, then the solution was made up to volume with the relevant solution.

2.1.6 Animals and Animal Tissues

Ideally, human tissue would be used for the diffusion studies, as this is the most relevant tissue type as these studies are to mimic *in vivo* human research. However, due to ethical restrictions porcine bladder tissue was used as a tissue alternative. This species has been shown to be a good mimic for humans as previous studies has compared the clozapine changes overtime in a variety of samples from pigs (Flanagan, Amin and Seinen, 2003).

Porcine (*Sus scrofa*) bladders: Obtained from local abattoirs (L Woods and Sons/N Bramall and Son), from approximately six month old large white cross pigs. The pigs were killed within the hour of receiving the bladders. The bladders were placed into a plastic bag and stored in a cool box with ice until arrival at the laboratory within the hour.

Rats (*Rattus rattus*): Medium, frozen, female rats were obtained from The Pet Warehouse in Huddersfield. The rats were stored in a cardboard box in a fume cupboard for one day to defrost. The average weight of the rats used in the 9-day experiment was 176.39g.

2.2 Methods

2.2.1 Preparation of biological tissue and animals

On arrival at the laboratory, the orientation of the bladder was noted before removal from the urethra. The bladders either remained intact (See Section 2.2.1.3), were sectioned into four (intra-bladder experiments) (See Section 2.2.1.2) or had one section removed (inter-bladder experiments) (See Section 2.2.1.1). After appropriate sectioning of the bladder tissue, the bladder pieces were immediately transferred to the Franz cell or frozen for one week before analysis. The tissues were individually removed from the bag used for transfer and cleaned before being mounted in order onto the Franz cell. Deionised water was used to clean the bladder section and absorbent tissue (blue roll) was used to remove any excess urine/faeces from the tissue, scissors were used to remove any connective tissue still attached to the outside of the bladder wall.

2.2.1.1 Inter-bladder studies

2.2.1.1.1 Fresh Bladders

The orientation of the bladder was recorded then a section was removed from the midpoint of the right side of the bladder. The bladder was opened out flat using a single vertical cut running from the top of the bladder to the base. The method for obtaining the middle of the bladder was to measure the length of the bladder from top to bottom using digital callipers (Absolute AOS Digimatic, Mitutoyo Callipers) and halving this value so the middle of the donor chamber was located. The lid of the Franz cell was placed to allow a template to cut the bladder to the correct size. The bladder thickness was then recorded using the digital callipers to determine if the thickness of the bladder varied and as a result had an effect on the rate of diffusion of the drugs through the tissue.

2.2.1.1.2 Frozen Bladders

The bladders were removed from the urethra and transferred to a labelled plastic bag with the date of collection. The bladders were then frozen at -7°C for one week before removing the day before the start of the bladder diffusion study to defrost to room temperature in a fume cupboard overnight. See section 2.2.1.1.1 for the bladder tissue preparation.

2.2.1.2 Intra-bladder studies

The intra-bladder experiments used the bladder orientation to allow the bladder sections to be mapped to determine any variation in diffusion from different parts of the same bladder. The bladder was sectioned into four (See Figure 2.2) using scissors and opened out using a single vertical cut from the top of the bladder, down one side to the base of the bladder. The Franz cell lid was used as a template to section the bladder into quarters. The lid was placed onto each section as to remove any overhanging tissue using scissors. The thickness of each section of the bladder was measured using digital callipers (Absolute AOS Digimatic, Mitutoyo Callipers).

2.2.1.3 Whole Bladder Studies

All experiments were carried out at room temperature (20°C). Rhodamine B (100 or 200 mg/L) was the compound added into the bladders for both full and half-filled porcine bladder volumes. The six bladders per

experiment were sealed with a cable tie at the top of bladder and secured to the 400 ml beakers with string. The solutions used to submerge the bladders were 20 mM phosphate buffer (PBS) at pH 7.4 and 20 mM ammonium acetate (AA) at pH 5. The volume added to each beaker was 300 ml. The setup was left to equilibrate for 30 minutes. Sample volume taken was between 4-5 ml, filtered using cellulose acetate syringe filters (Chromacol, 17 mm, 0.45 µm), then centrifuged at 3000 rpm for 5 minutes if not a clear sample. One sample from each bladder was taken per day over 5 days. The mean concentration of rhodamine B was determined in triplicate using a UV spectrophotometer (554 nm) (See Figure 2.1). Experimental parameters are shown in the table below:

Table 2.1 Whole bladder experimental parameters

Experiment	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
pH	7.4	7.4	7.4	7.4	5	5	5	5
Concentration (g/L)	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2
Volume	Full	Full	Half-Full	Half-Full	Full	Full	Half-Full	Half-Full

2.2.1.4 In vivo rat study

A medium sized frozen female rat was thawed and opened using a scalpel (Swann Morton stainless steel surgical scalpels) to reveal the bladder and catheterised using a 24G IV catheter (Briar Dawn Veterinary Surgery). Silver nitrate (1M) solution, the contrast medium used for the Micro-CT, was taken up into a 1 ml syringe (1 ml Terumo syringe without needle) and dispensed into the bladder using the catheter. The outside of the urethra was tied off with cotton string. The outside of the urethra and the midline incision was sealed with superglue to keep the organs intact. The rat was then secured to a polystyrene sheet with string and placed inside a plastic weapons tube that was sealed (See Figure 2.3). Between analyses, the rat was stored horizontally at room temperature to mimic an individual that had died indoors.

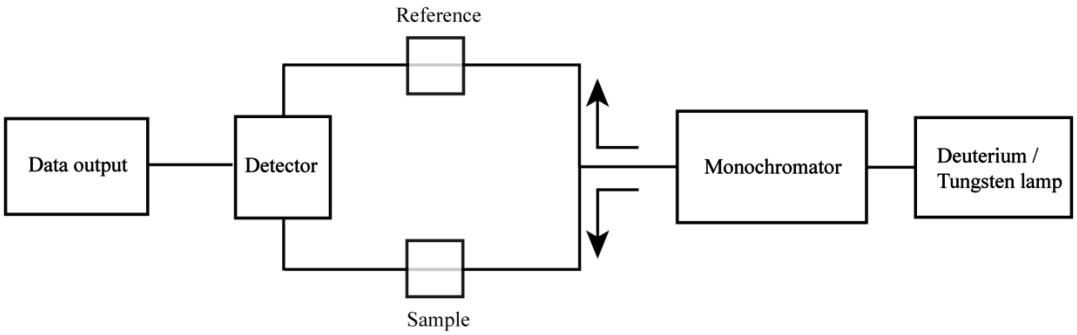

Figure 2.1 Schematic of a single-beam spectrophotometer

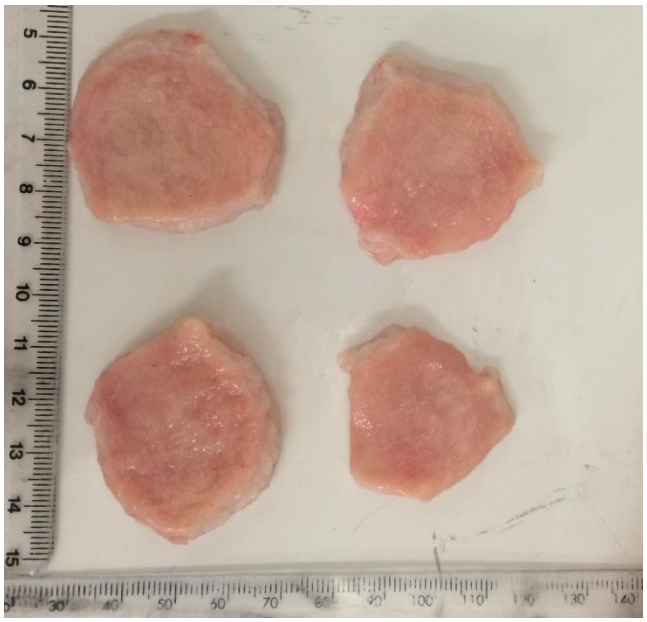

Figure 2.2 Example of a bladder sectioned for an intra-bladder diffusion study at 20°C in PBS pH 7.4

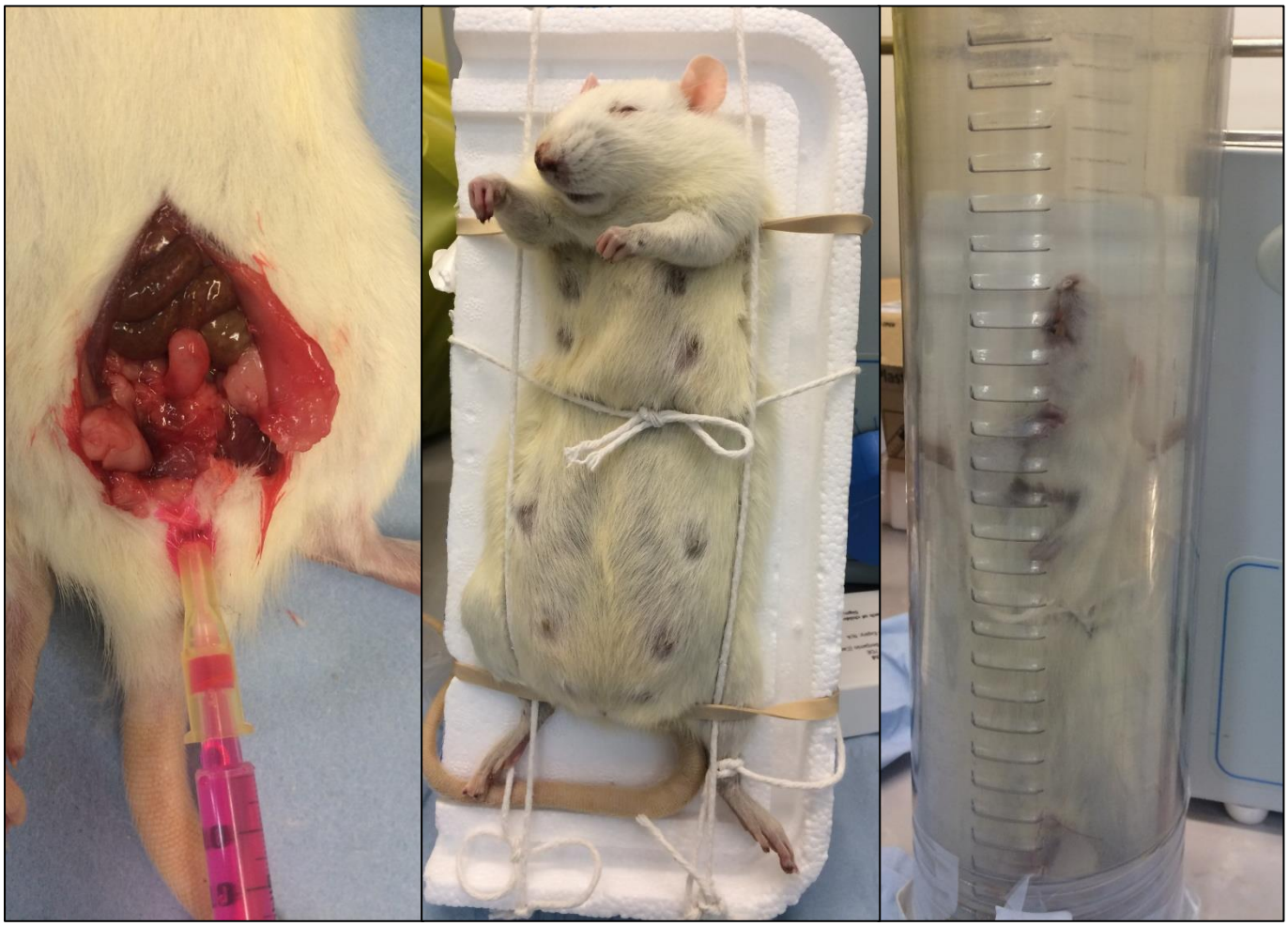

Figure 2.3 Set-up of the rat used for the *in vivo* study analysed on the Micro-CT

2.2.2 The use of Franz-Cells to Study diffusion from the Bladder

2.2.2.1 Introduction to Franz-Cells

In order to measure the diffusion of both drugs and the model compound Franz cells were utilised. This experimental technique used two chambers separated by the insertion of a membrane (See Figure 2.4). The donor chamber contains the compound of interest and the acceptor chamber has a sampling port for removal of solutions for analysis. There are different types of Franz cell, dependant on the type of experiment carried out, including in-line or flow-through cells (Mah *et al.*, 2013), side-Bi-side cells (Suhonen and Pasonen-Seppänen, 2003) and static Franz cells (Zhang, Liu and Du, 2009). These cells can also be temperature controlled with water jackets around each individual cell (Wang *et al.*, 2007) and also protects the diffusion cells from light (Deneer *et al.*, 2002). The use of the static Franz cells rely on gravitational forces as well as the passive diffusion of the drugs, these cells are used to mimic *in vivo* situations (Friend, 1992). The main function is to determine physiochemical properties of compounds via diffusion through a membrane. They have been used to calculate the rate of absorption, total absorption (Franz, 1975), efficacy (Sahoo, Ma and Labhasetwar, 2004) and osmolality (Wang, Zuo and Chow, 2009). Comparison with *in vivo* data has shown correlations, which result in diffusion modelling experiments using this equipment to mimic real life situations (Wagner *et al.*, 2002). Another parameter included the ionisation state where two drugs were analysed in two different pH solutions including a permeation enhancer, diffusing through porcine buccal tissue. The results showed the permeation enhancer increased diffusion of the ionised form of one drug, however it did not affect the other drug (Deneer *et al.*, 2002). Skin from different species can be directly compared as shown by a study comparing human to rat skin in relation to permeation (Suhonen and Pasonen-Seppänen, 2003). In addition, models can be produced for compounds to compare theoretical data to experimental data, which has had some success, as there were general result correlations. However, there were limitations to the model as not every parameter was included, mainly involving the simulation being based on a simplified version of the skin membrane. The actual skin can be divided into at least two different layers, which would affect the values for the partition coefficients and diffusion. In addition, the effects from binding was not taken into consideration (Naegel and Hahn, 2011).

Advantages of this *in vitro* method show linear correlations between *in vitro* permeation of compounds through human skin and *in vivo* permeation detected using Raman spectroscopy (Mohammed *et al.*, 2014). Another advantage is the specificity of the experiments, after the diffusion study the layers of the tissue can be separated by a method of tape stripping (Netzlaff, Kostka, Lehr, 2006). This method involves adhering adhesive tape to the topmost layer of skin, applying force with a fingertip and repeatedly removing the tape (Dreher *et al.*, 1998; Naegel and Hahn, 2011). This allows for the diffusion of the compound to be isolated in individual layers of the tissue to determine how the compound diffuses through tissue, including preferential diffusion through certain tissue layers (Creel, Lovich and Edelman, 2000). In addition, as aspects of the setup can be controlled, agitation of the receptor chamber can reduce the unstirred boundary layer under the membrane that would result in limiting diffusion, which could affect the overall drug diffusion (Friend, 1992).

A possible disadvantage of investigating previously living tissue is during the experiment the excised tissue could undergo decomposition, which could increase the drug diffusion through the degrading tissue. This has been shown with mice skin after approximately 4.5 hrs when comparing *in vitro* to *in vivo* methodologies (Venter *et al.*, 2001). As a result, this could restrict clinically based experiments on the Franz cells. However, as this research is trying to mimic the decomposition after death this study showing decomposition occurring *in vitro* would not be a disadvantage in this case. In addition, the shape of the receptor chamber restricts complete mixing, mainly in the sampling port and the portion underneath the membrane. Studies show complete mixing can take up to 30 minutes. Furthermore, with biological tissues it is difficult to maintain a constant water content in the receptor chamber, as if there is no equilibrium between solution and tissue, water could be taken up into the tissue affecting the concentration in the receptor chamber (Friend, 1992).

The Franz cell method chosen for this research seems to be a good model for the parameters that were being investigated. This model has been used previously with success with a range of different tissues, showing that this equipment is suitable for bladder tissue. The static Franz cell will allow the drug to only use the method of passive diffusion under gravity to travel through the tissue.

2.2.2.2 Experimental Set-up of Franz Cells

The Franz cell set up consisted of a water bath (Fisher Circulator Iso 4100 R20, 6 litres) and the Franz-cell diffusion chambers (See Figure 2.10). The dimensions of the Franz cell with the flat ground joint was a joint inner diameter of 20 mm and outer diameter of 30 mm, 15 ml receptor volume and 5 ml donor volume, 3.14 cm^2 orifice area and a jacket diameter of 30 mm. The bladder thicknesses ranged from 1.5 – 9.0 mm. The Franz cell set up (PermeGear V6A-02) consisted of six clear, glass, Franz cells mounted onto a stand with magnetic stirrers and 6 clamps (See Fig 2.5), needles (See Fig 2.7) and donor chambers (See Fig 2.6). A water bath was attached to the Franz cell (See Fig 2.9). Before the mounting of the bladder sections, the water bath was set to the relevant temperature (37, 20, 5°C) and allowed to equilibrate for at least 2 hrs before use. Following appropriate dissection of the bladders, (see section 2.2.1) the sections were briefly washed with distilled water. In order to mount the bladder sections the ends of a u-shaped paperclip pierced each side of the bladder tissue to hold the tissue taut for mounting onto the Franz cell (See Fig 2.11). The support ring (See Fig 2.8) was placed on the top of the acceptor chamber of the Franz cell, the tissue was placed on top of this (lumen facing upwards) and then the second support ring (See Fig 2.4). Two strips of Parafilm were placed in-between the clamp. The glass donor chamber was placed on top of the support ring and secured with the clamp. The paperclip was removed from the bladder tissue. Each section of Parafilm (Sigma, UK) was stretched around the chambers individually overlapping each side for a full seal between the two cells.

Approximately 2 ml of PBS or AA was added to each acceptor chamber using the 12 ml plastic syringe (Syringe Luer-Slip disposable, Fisher brand) and needle through the spout. The first chamber was tilted upside down to check there was no leakage out of the donor chamber. As the chamber was still in this position, the relevant solution (PBS or AA) was added to the acceptor chamber (15 ml) using a 12 ml syringe and needle. Once the solution covered the bladder tissue it was not moved until the chamber was full. If

there were air bubbles present in the bottom of the chamber, it was slowly tilted upright until the air bubbles reached the neck of the spout. Once removed, the chamber continued to be filled until the solution travelled up the spout. The chamber was then tilted upright and replaced into the holder. The acceptor chamber solution was filled to the 15 ml line marked on the spout. The end of the spout was sealed with Parafilm. This step was repeated for each chamber (See Figure 2.12). The magnetic stirrer was switched on for each Franz cell. The donor chamber was sealed with Parafilm to minimise the evaporation of the solution (AA or PBS) solution. To allow for equilibration the tissues were left for 30-45 minutes. After equilibration, the donor chamber was filled with 100 mg/L rhodamine B or mixed antidepressant drug solution, which holds 5 ml of solution. After the first sample was taken then the chamber was re-sealed with Parafilm. Once the Franz cells were set up they were not moved for the rest of the experiment, as the movement will displace the solution from the tissue, which would cause disruption in the diffusion of the drug from the tissue to the solution. The water bath was kept at a constant temperature, the magnetic stirrer was rotating at 500 rpm throughout the entire experiment, and after each sampling the Parafilm was replaced onto the spout to prevent evaporation of the solution from the acceptor chamber.

Figure 2.4 Image of a Franz Cell

Figure 2.5 Franz cell Clamp

Figure 2.6 Franz cell donor chamber

Figure 2.7 Franz cell needle

Figure 2.8 Franz cell support ring (greaseproof liner)

Figure 2.9 Water Bath (Fisher)

Figure 2.10 Photograph of the Franz-Cell setup showing the water bath (background), stirring plate and diffusion chambers

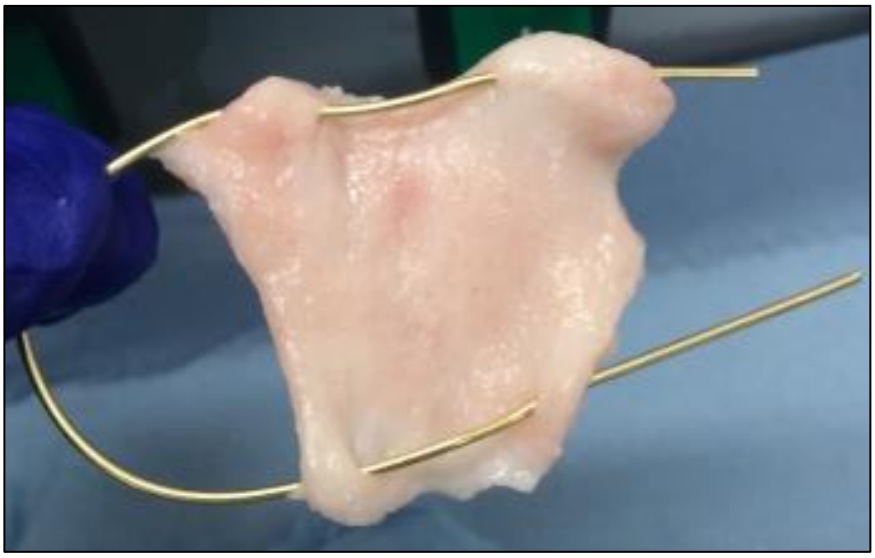

Figure 2.11 Photograph of a bladder section secured onto a paperclip for mounting

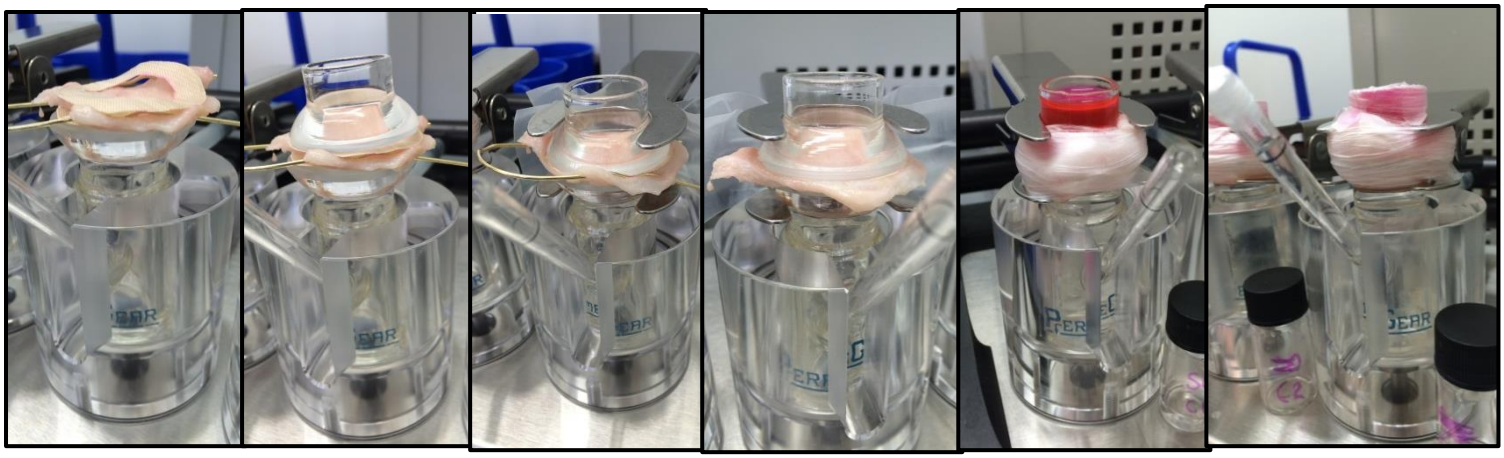

Figure 2.12 Sequence of mounting the bladders onto the Franz cell

2.2.2.3 Sampling from the Franz-Cells

A sample was taken from the acceptor chamber immediately after adding the donor chamber solution for a time zero reading. The 1 ml plastic syringe (1 ml Terumo syringe without needle) and metal needle were used to sample the acceptor chamber using the spout removing approximately 0.5 ml. The sample was immediately filtered using a cellulose acetate syringe filter (Chromacol, 17 mm, 0.45 µm) and transferred into an 8 ml glass vial. The time was recorded for when the sample was taken to allow a time interval to be established. Fresh blank solution (PBS or AA) immediately replaced the lost volume, using a separate needle and plastic syringe and the spout was resealed using Parafilm. The rhodamine B samples were analysed on the UV and the mixed antidepressants were analysed on the HPLC, the solutions were discarded after analysis. A sample was taken from each chamber twice on the first day (immediately after experiment setup, then approximately 2-3 hrs later), then three times a day (morning, afternoon and evening) over 5 days. Three samples a day were taken to show any change in the diffusion throughout the day and these values were compared with the other 4 days to show intra-day and inter-day variation.

2.2.2.4 Cleaning the Franz Cells

In order to prepare the Franz cells for reuse after the end of the experiment the chamber setup was dismantled with the donor chamber and support rings removed for cleaning with 1 ppm trigene (Trigene advanced) and deionised water.

2.2.3 Measurement of rhodamine B Concentration using UV-Visible spectrometer

2.2.3.1 Introduction to UV

Wavelength (λ) is the unit used in ultraviolet (UV) spectroscopy to measure the energy of photons and is measured in nanometres (nm). The UV region is between 200-400 nm and visible range extends from 400 to 800nm. The amount of absorption at a monochromatic wavelength is determined by two laws of absorptiometry. This related the incident light (I_0) to the transmitted light (I) (Figure 2.13). The two laws are Lambert's Law (Lambert, 1760) and Beer's law (Beer, 1852); these are combined to become the Beer-Lambert law (Parnis and Oldham, 2013), which relates the path length and absorption to the concentration.

$$log \frac{I_o}{I} = \varepsilon cl$$

I_0 = incident light, I = transmitted light, Ɛ = molar absorption coefficient, C = concentration, l = path length (cm). The Beer-Lambert law has limitations including the solvent effects; different solvents could affect the analyte of interest. These solvents include pH based solutions as ionisable analytes conjugate to differing extents dependant if the analyte is in the ionised or non-ionised form. There could be absorption at higher wavelengths if there was more conjugation (Negrusz, 2013). In relation to this work, there are two pH based

solutions that are involved and the drugs have pKa values showing there could be variation in the ionisation dependant on pH, which may affect the amount of the drugs diffusing through the bladder tissue.

Ultraviolet (UV) and visible spectroscopy is a technique that allows for the quantification of a substance through the absorbance of light. The types of substances are usually limited to those possessing a chromophore, which is the conjugated functional group responsible for producing a visible result on the UV e.g benzene ring (Negrusz, 2013). Quartz cells are used for most analysis as they are more reliable with limited variation between cells and have a higher transmittance of radiation than plastic cells between the wavelengths 190-1000 nm (Negrusz, 2013). In single-beam spectrophotometers, the results are achieved by focussing UV radiation through the sample of interest, this is a narrow beam achieved by a monochomator that separates the radiation to obtain single wavelengths from the UV lamp (Negrusz, 2013) (Figure 2.1). As the beam passes through the sample some of the radiation will be absorbed by the compound. This energy may then be emitted in numerous ways, most useful is the emission of radiation of a longer wavelength that can be detected using instrumentation (Flanagan, Taylor, Watson, 2007). The amount of radiation that has been transmitted through the sample, in comparison with a set of calibration standards under the same conditions, determines the absorbance. Then through calculations, the concentration of the substance in the sample and the absorbance is linearly related to concentration (Levine, 2013). This technique has been used to detect a model compound used to highlight changes in bladder tissue including indigocarmine (Monson *et al.*, 1991) and rhodamine B (Skopp *et al.*, 1997).

2.2.3.2 Sample analysis on the UV

The samples were run on the UV-Visible spectrometer (Agilent, Cary 60) using a 400 µl volume quartz cuvette (Hellma Analytics, high precision cell, synthetic quartz light path 10 mm). Photometric software was used as only one wavelength was being used to detect the rhodamine B. The wavelength was set to 543 nm. The cuvette was filled with blank solvent (PBS or AA) was placed into the UV-Visible spectrometer and the instrument was blanked. In order to stop any possible error in readings due to fingerprints or other contamination the cuvette was rinsed with deionised water and dried with absorbent tissue in-between samples. The samples were analysed in triplicate using the same cuvette to obtain an average result, discarding the sample after analysis.

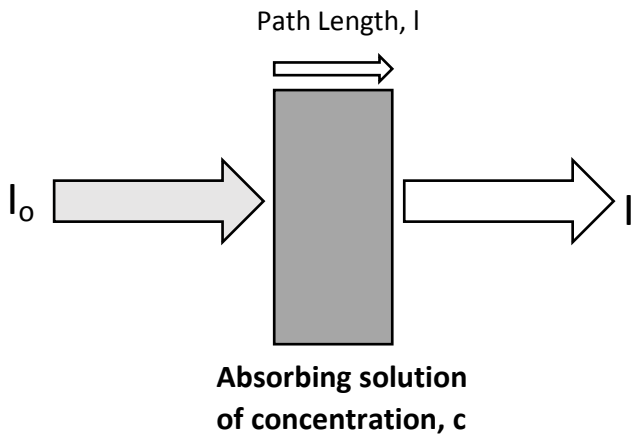

Figure 2.13 Path of a UV radiation beam through a sample (Diagram based on (Medicinescomplete, no date) and generated by the author)

2.2.4 Measurement of Antidepressant Drug Concentration using High-Pressure Liquid Chromatography with a Diode Array Detector (HPLC-DAD)

2.2.4.1 Introduction to HPLC

Chromatography is a century old, robust technique used for separation of compounds using two phases, usually a stationary and mobile phase. The separation occurs when a mixture of compounds within the same sample react differently in the stationary phase, this results in the compounds eluting from the column at different times (Robards, 2004). High performance liquid chromatography (HPLC) is a type of chromatography, specifically reverse phase HPLC that involves a polar mobile phase, and a non-polar stationary phase. The compounds travel through the stationary phase dissolved initially in a sample solvent, which then mixes with the liquid mobile phase. The compounds are constantly being retained and released along the stationary phase. The amount of retention depends on a number of compound properties including molecular size and the composition of the mobile phase including polarity (David, 2013). A chromatogram is the graphical representation of the HPLC results, which shows the response of the detector over time (Levine, 2013).

The stationary phase usually contains octodecylsilyl bonded silica particles and retains non-polar compounds, which is the usual state for drug compounds, for longer periods; as a result, these compounds would have a longer retention time. The retention time is an important element as the drugs need to interact with the stationary phase to some extent to allow for the detector to notice the analytes. Each drug is visualised on the chromatogram by a peak and the shape of the peak needs to be optimised for a successful identification or quantification. Peak width depends on the separation of the compounds from each other, the narrower the peaks the better separation and therefore resolution (David, 2013). Resolution is a parameter with no units that needs to be satisfied for acceptable results; it is the difference in the retention times between two compounds. An acceptable value is 1.5, which shows that there is acceptable distance between the two compound peaks (Levine, 2013). The peak heights depend on the amount of compound present in the samples and the sensitivity of the instrument to the compound. This technique is able to identify and quantify a wide range of compounds from a mixture (David, 2013).

The HPLC can be split into parts including pumps, injector, column, and detector (See Figure 2.14):

Pumps:

There is usually two mobile phases or eluents that are mixed together to form a specific composition that is complementary to the stationary phase and the compounds being detected. The first eluent contains an organic solvent including acetonitrile or methanol and possibly a buffer for pH stability (David, 2013). There are two main elution methods: isocratic and gradient. The isocratic method has a constant composition of the eluents, and the gradient has pre-programmed alterations to the composition of the eluents. Gradient methods usually have longer run times as the eluent composition at the end of the run is different from the start and the method has to re-equilibrate before the next sample is run. The isocratic systems are mainly

used for a small number of analytes, where gradient systems are used when there is a wide range of types of compounds in the samples resulting in longer run times (Flanagan, Taylor, Watson, 2007). The pump system introduces the eluents into the HPLC, through all components including the injector, column, and detector, at the flow rate set by the method at a reproducible rate. Due to resistance from the column, as it is packed with particles, the pumps have to operate at high pressures to enable the solutions to reach all aspects of the HPLC system (David, 2013).

Injector:

The injector introduces the sample into the HPLC, to mix with the mobile phase before the solution enters the column, at reproducible volumes (David, 2013). The sample valve, which operates the introduction of the samples, has two variations. The internal loop allows volumes of < 1 µl to enter the HPLC system and the external loop that allows volumes from 2-3 µl to 20 ml. These injector loops are usually automated and are setup to inject a set of samples from a pre-programmed sequence of samples from the HPLC software (Flanagan, Taylor, Watson, 2007).

Column:

The column is the stationary phase and can be various lengths and diameters and also packed with a wide range of different particles dependant on the use of the column (David, 2013). The particle size can vary between 3-10 µm (Flanagan, Taylor, Watson, 2007), the most common particle type is porous silica based with alterations to the surface of the particles that allow for the different uses of the columns (David, 2013). This includes creating, C_8 and C_{18} columns, which are mostly used for general analysis in toxicology (Flanagan, Taylor, Watson, 2007). There are different types of liquid chromatography including normal and reverse phase, the first requires a polar stationary phase and a non-polar mobile phase and the latter is opposite. This requires different mobile phases dependant on the polarity of the stationary phase (Robards, 2004). To protect the column from the build-up of contaminants still present from the eluent or extracted samples a filter is placed in front of the column. These particles could damage the column and so the filter or guard column catches these particles before they pass onto the column (Levine, 2013). The column is placed into the column oven that can be set to specific temperatures dependent upon the method to improve peak shapes (Flanagan, Taylor, Watson, 2007).

Detector:

The detector is used to recognise when the composition of the mobile phase changes, which would be due to the elution of an analyte and convert this into an electrical signal. This in turn is corrected by the data acquisition system into a visual format showing the analyte (Meyer, 2010). The decision on which type of detector to use depends on the type of analytes being used as each is used to detect an element of the analyte that is not exhibited by the rest of the solution including the mobile phase (Robards, 2004). The different types of detectors include electrochemical, fluorescence (Robards, 2004), Fourier-transform infrared spectroscopy (Meyer, 2010) and UV/Vis (Robards, 2004). Both direct and indirect detection are available, the first is where the background signal of the mobile phase is low and the signal from the analyte can be

separated from this. The latter is the opposite where the signal decreases when the analyte is eluted from the column as the mobile phase has a high signal (Robards, 2004).

Fluorescence is based on the detection of the radiation emitted from excited electrons; there is a two stage process of absorbing radiation and then emitting a photon that is deemed fluorescence. Fluorescence detection can be sensitive however, it is very selective due to a number of solvents that could interfere with the signal and some analytes would have to under derivatisation to increase the sensitivity of this technique to the analyte. Electrochemical detectors use electrodes and detect the current produced when an electrical potential is applied to the analyte. However, there is only a limited range of electrodes that are available, limiting the different types of experiments able to be run on these instruments (Robards, 2004). Fourier-transform infrared spectroscopy results in an IR spectrum of the analyte, using a volatile mobile phase, a drawback of this detector is that is can be less sensitive in relation to other forms (Meyer, 2010). The most common type of detector is the UV detector; different variations of this detector are fixed and variable wavelengths and diode array. The diode-array detector is used to focus energy that is not yet diffracted through the sample, the detector then diffracts the energy onto a series of sensors that create a snapshot of the contents of the samples at that moment. These data is then converted into an electrical signal or a peak enabling the results to be compared to a database within the HPLC software to identify the peak (Levine, 2013).

HPLC is one of the main techniques used in toxicology for identification and quantification of compounds from post-mortem cases (Hale, 1998). It is also still used in the research sector detecting a wide range of compounds used in diffusion studies (Deneer *et al.*, 2002; Moch, Salmon and Armesto, 2014).

2.2.4.2 Sample analysis on the HPLC-DAD

The mobile phases were both degassed via vacuum filtration (Phenomenex, Phenex filter membranes, 0.45 µm, 47 mm, Nylon). The HPLC (Dionex Ultimate 3000 UHPLC, Thermo, UK) was equilibrated before running samples by changing over to the relevant mobile phase (See Sections 2.2.4.2.1 and 2.2.4.2.2), altering the mobile phase composition on the HPLC. The flow rate was increased to the method flow rate and purged for 5 minutes. After purging, the system was left for half an hour running the mobile phase at the relevant flow rate to clean out the column and flush the column of any solvent from previous analyses. The samples from the Franz cells (See Section 2.2.2.3) were individually transferred to plastic 250 µl HPLC vials (Agilent, Vial, crimp/snap top, polypropylene, 250 µl) using a 3 ml plastic transfer pipette (Fisher, Pipette 3 ml 153 mm length Pasteur non-sterile x 0.5 ml).

The sample sequence was: blank water, blank PBS/AA, calibration (0.156 mg/L – 10 mg/L), blank PBS/AA, Sample 0 (Bladder 1-6), blank PBS/AA, Sample 1 (Bladder 1-6) and this was repeated until Sample 13 (Bladder 1-6). The HQC sample was at the end of the sequence to check the bladder samples did not affect the column (system suitability test) and a blank methanol was analysed as the internal standard in the calibration was dissolved in methanol.

2.2.4.2.1 PBS pH 7.4

HPLC parameters included the use of a Waters Spherisorb 5 µm OD/CN, 4.6 x 150 mm Analytical Cartridge. The mobile phase consisted of the organic phase (70% acetonitrile, 27.5% deionised water, 2.5% triethylammonium phosphate buffer (TEAP)) and the aqueous phase (97.5% deionised water, 2.5% TEAP). The method type was isocratic with the mobile phase composition of 57% organic: 43% aqueous. The flow rate was 2.0 ml/min, with a column temperature of 25°C. The sample run time was 5 minutes and the diode array detector was set to 210 nm.

2.2.4.2.2 pH 5

HPLC parameters included the use of a Phenomenex Gemini 3 µm C_{18} 110Å 150 x 4.6 mm column. The mobile phase consisted of the organic phase (50% acetonitrile: 50% methanol) and the aqueous phase (20 mM ammonium acetate). The method type was isocratic with the mobile phase composition of 70% organic: 30% aqueous. The flow rate was 1.25 ml/min, with a column temperature of 30°C. The sample run time was 5 minutes and the diode array detector was set to 210 nm.

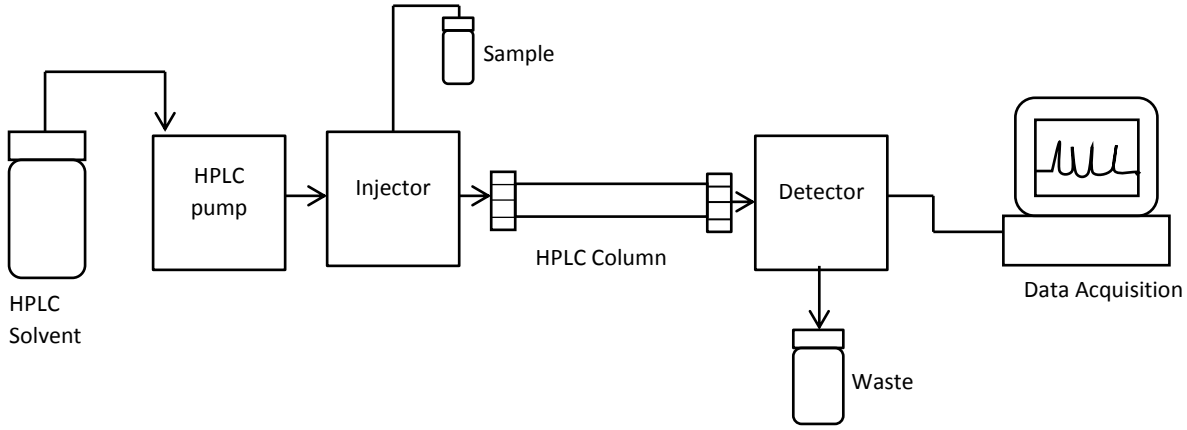

Figure 2.14 HPLC Set-up (Diagram based on (Giri, 2015) and generated by the author)

2.2.5 Scanning of the silver nitrate infused rat on the Micro-CT

2.2.5.1 Introduction to X-Ray Computed Tomography

Computed Tomography (CT) is an imaging technique that uses x-rays to display 3D images of biological tissues (Hrvoje, Lusic, 2014). It is mainly used in medicinal imaging for cancer in numerous areas within the body including the intestines (Antoch *et al.*, 2004) and more recently whole-body scans (Meier, Hamill, Jones, 2017). However, it has begun to be adopted in the autopsy process aiding in gathering evidence in drug related fataliities (Rohner and Franckenberg, 2013; Winklhofer, Surer and Ampanozi, 2014). It reduces the risk of exposures to biohazardous materials (Winklhofer, Surer and Ampanozi, 2014) as a non-invasive and non-destructive technique (Hrvoje, Lusic, 2014).

One type of electromagnetic radiation is x-rays, numerous x-rays form a 3D CT image. The image is produced by using a cathode and an anode, usually tungsten-alloy, and accelerating electrons between the two through a vacuum tube. Initially, the CT had poor contrast between biological tissues, which resulted in the use of contrast media being introduced (Schambach, Bag and Schilling, 2010). CT imaging using contrast media improves the resolution of the image as the solutions used have a higher density than the surrounding environment, which could include organs or blood vessels. The high density allows for better absorption of the x-rays (Hrvoje, Lusic, 2014). Studies have compared different contrast media in relation to post-mortem CT (PMCT). The results show that hydro-soluble solutions would diffuse out of the vessels after a long PMI due to degradation of tissues (Grabherr and Grimm, 2015). As a result, further investigations were carried out to include a mixture of a water-soluble compound and polyethylene glycol (PEG), as this has oily properties that would limit diffusion from vessels (Ross *et al.*, 2008). The contrast media can be introduced into the individual either via ingestion or intravenously. These media usually include barium or iodine in medically related investigations as these elements possess a high atomic number (Foley, Ghahremani, 1982; Hrvoje, Lusic, 2014). Silver has been used commercially due to anti-microbial activity and has been previously used in living animal studies to determine the clearance of silver from the system. The results show that silver distributes in most organs and does not harm cells (Lee and Kim, 2013).

There are two types of Micro-CT, the first is where the object is stationary, and the x-ray source is moved around the object. The second type of CT, mainly used in *in vivo* animal research, is where the x-ray source is stationary and the object itself moves the 360° on its own axis (See Figure 2.15). During this time a number of projections are recorded, then software would render the images into a 3D reconstruction of the object (Hrvoje, Lusic, 2014).

Micro-CT is a more recent technique that can image small animals with contrast media producing high quality 3D images in a shorter time (Ghanavati and Yu, 2014). The initial research focussed on bones including bone density (Engelke, Karolczak, 1999) and bone diseases including osteoporosis (Toga and Thompson, 2001). The improvements in CT resolution allowed for more detailed research into using contrast media to highlight

the vasculature in different parts of mice including the kidney (Bentley *et al.*, 1998) and the brain (Ghanavati and Yu, 2014).

This previous research mainly concentrated on living animals, with limited research into *in vivo* post-mortem breakdown of organs. However, CT scans have been previously carried out on rats to determine any changes that occur between an anesthetised and dead rat. The rats were laid flat in the CT scanner. There were no observable changes between anesthetised and dead rats, which were not supported by a body mould. However, there were changes to tissues associated with post-mortem changes to organs. The urinary bladder could be identified ante-mortem and post-mortem, with the only change of the bladder being voided due to death (Lapin and Allen, 1997). In addition, silver nanoparticles dissolved in deionised water were introduced into the ear canal of a rat to determine any post-mortem movement. The solution could be detected down to concentrations of 37.1mM and visible up to 7 days post-mortem (Zou *et al.*, 2015). Furthermore, silver nitrate has been previously used successfully with mice (Raj *et al.*, 2014) and humans (Watz, Breithecker, Rau, 2005) in CT based research.

2.2.5.2 Sample analysis on the Micro-CT

The diffusion of silver nitrate from the rat bladder was analysed using a Micro-CT Scanner (X-Tek XT H 225) (See Figure 2.16). The analysis parameters were 1583 X-Rays, 500ms exposure, 45 minute run time, 110 µamps current and 130 Kv voltage. During the analysis, the rat was vertically secured to a plate inside the CT scanner. The first scan was immediately after preparation, and then the rat was laid flat at room temperature (approximately 20°C) and scanned six times over 9 days. The analysis times included day 1, 2, 6, 7, 8 and 9.

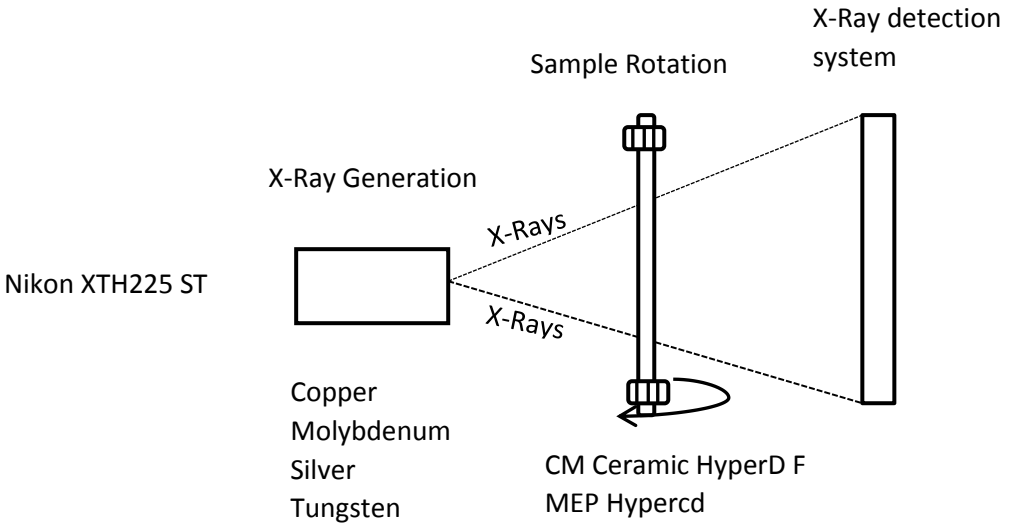

Figure 2.15 Schematic Diagram of a Nikon XT H 225 ST CT scanner

Figure 2.16 X-Tek XT H 225 CT Scanner used for the *in vivo* rat study

2.3 Data Analysis

Statistical analysis was carried out on the data sets to determine the concentration of the drugs that diffused through the tissue. Further analysis included determining if the concentrations were significantly different dependant on the parameters that were changed in the experiments. The software packages included in these processes included Chromeleon 6 (Thermo Scientific, UK), Excel 2010 (Microsoft), and GraphPad Prism 6 (GraphPad).

2.3.1 Microsoft Excel

Excel spreadsheets with a single-factor ANOVA validation were setup and used to validate all three compounds used in this research (rhodamine B, amitriptyline and nortriptyline). In addition, all the bladder diffusion study results were recorded in Excel 2010 calculating the cumulative concentrations of the compounds diffusing through the bladder tissue. The concentrations were calculated in Excel 2010 using the calibration lines produced each day from the set of freshly prepared calibration standards run with the solutions from the diffusion studies.

Excel 2010 was also used to calculate the flux values over the course of the experiment using the calculated concentrations from the bladder diffusion studies. The concentrations were converted to mass and this was used to calculate the flux using the time, sample volume values and diffusional area (3.14 cm^2) from the experiments, using the following formula (Brodin, Steffansen and Nielsen, 2010):

$$Flux = \frac{cumulative\ mass\ (mg)}{diffusion\ area\ (cm^2) x\ time\ (\text{minutes})}$$

The flux values were then averaged to calculate the permeability of rhodamine B at each set of parameters investigated using the bladder studies. The permeability was calculated in two different ways dependant on the results, steady state conditions were met with < 10% of the donor chamber diffusing through to the acceptor chamber. A non-steady state condition was introduced when the diffused concentration increased above 10% of the original donor chamber concentration. The calculations, shown below, were used from Birger diffusion paper (Brodin, Steffansen and Nielsen, 2010). The permeability was calculated then converted to cms^{-1}.

$$Permeabiliy\ (Steady\ state) = \frac{Average\ Flux, J\ (mgcm^{-2}min^{-1})}{Donor\ concentration\ (mg/L)}$$

$$\underline{Permeability\ (non-steady\ state):}$$

$$Equation\ 1:\ C_\infty = \frac{mass\ (t=0)}{V_{donor} + V_{receiver}}$$

$$Equation\ 2:\ \frac{C_\infty - C_{receiver,t}}{C_\infty} = e^{-kt}$$

$$Equation\ 3: k = AP\frac{V_{donor} + V_{receiver}}{V_{donor}V_{receiver}}$$

C_∞ = Final concentration in both of the compartments (mg/L)

Mass (t=0) = Mass at time zero (initially added to the donor chamber) (g)

V_{donor} = Volume of the donor chamber (ml)

$V_{receiver}$ = Volume of the receiver chamber (ml)

$C_{receiver,\ t}$ = Concentration of the compound at time t (mg/L)

K = Constant (min^{-1})

A = The area of the tissue (cm^2)

P = Permeability ($cm\ s^{-1}$)

In addition, Excel 2010 was used to calculate the differences between the original donor chamber concentrations and the combined final concentrations in the donor and acceptor chambers for all the bladder diffusion studies using all three drugs.

2.3.2 Chromeleon 6.0

Chromeleon 6, the HPLC software package, calculated the amount of drug present in the standards using peak height.

2.3.3 GraphPad Prism 6

This statistical software was used to determine if there was a significant difference between the different temperature, pH, concentration, and bladder volume data sets.

The bar charts in both Chapter 4 and Chapter 5 included stars above the bars to show the magnitude of the significant difference from the mean value. The stars represent the numerical representation of the significant difference, which is the p value (See Table 2.2). The smaller the p value, equal to or below 0.05, the larger the significant difference between sets of data, which results in rejecting the null hypothesis. The null hypothesis states there is no significant difference between data sets. Therefore if the null hypothesis is rejected, shown by a small p value, this means there is significant difference between data sets (*GraphPad Prism 6 Statisitcs Guide*, 2015). The statistical tests are described in sections 2.3.3.1 and 2.3.3.2.

Table 2.2 Label meanings for the statistical bar charts (*GraphPad Prism 6 Statisitcs Guide*, 2015)

Label	Description (P value)	Significance
NS	No significant difference	-
*	0.01 - 0.05	Significant
**	0.001 - 0.01	Very Significant
***	0.0001 - 0.001	Extremely Significant
****	Less than 0.0001	Extremely Significant

2.3.3.1 Chapter 4 Statistical Analysis

The statistical tests used for the Chapter 4 temperature, pH and degradation results was a Two-Way ANOVA with the simple effects within row multiple comparison. The post-test used was the Bonferroni as only one cell

mean comparison was needed, the comparison between the cell means within the same row. As comparing the difference between a parameter (cumulative concentrations) at a specific time point.

The statistical analysis on the results from the bladder studies involving the two drugs (amitriptyline and nortriptyline) had missing data. Therefore, the data could not be compared at set time points resulting in Two-Way ANOVA being unsuitable to analyse the data. As a result, the data were grouped by the respective solutions (PBS and AA) and an unpaired t-test was carried out to determine if the two sets of data were significantly different. The p value from the F test within the prism results was used to determine if the results were significantly different, which would be with a value equal to or below 0.05.

The inter-bladder thicknesses were plotted on a histogram to determine the spread of bladder thicknesses throughout all the diffusion studies. Furthermore, a comparison between fresh and degraded bladder tissue thicknesses was calculated using an unpaired t-test with Welch's corrections as did not want to assume both sets of data had the same standard deviation. Also, carried out a two-tailed t-test to determine if there was any significant difference above or below the mean value.

2.3.3.2 Chapter 5 Statistical Analysis

The statistical tests used for the Chapter 5 concentration, bladder volume, and pH results were a Two-Way ANOVA with the simple effects within row multiple comparisons. The post-test used was the Bonferroni as only one cell mean comparison was needed, the comparison between the cell means within the same row. As comparing the difference between the cumulative rhodamine B concentrations at a specific time point.

2.3.4 X-Tek 3D Pro

The software used with the computed tomography (CT) enabled the images to be stitched together to form a 3D rendered image that could be rotated in any direction to identify the bladder location. In addition, layers from the image could be removed so only the skeleton and the CT drugs could be identified.

Chapter 3: Method Development and Method Validation

3.1 Introduction

The validation of methods is the process of determining if the method is 'fit for purpose', if the method would produce accurate and precise results when used in routine analysis. There are a number of factors that are taken into consideration when validating a method including a calibration range, linearity, accuracy and precision, drug stability, limit of detection and quantification and selectivity (Flanagan, Taylor, Watson, 2007). The Forensic Toxicology Council produced a document, named the Scientific Working Group for Forensic Toxicology (SWGTOX) validation guidelines (SWGTOX, 2013). This document outlines the standard guidelines for validating a method used in the field of forensic toxicology.

The main factors that could have an effect on either the method or the analytes have been included to cover the main process that samples undergo in a toxicology laboratory. Initially, a validation plan is suggested to determine the parameters expected for the method to be validated, which includes determining values for each stage of the validation. There are different levels of validation dependant on the use of the method and the instrument used, as a screening method would not need the use of calibration standards and ionisation suppression/enhancement would not be required for HPLC-DAD methods. The validation samples used would be fortified samples prepared specifically for the set of validation experiments (SWGTOX, 2013).

A quantification method would use most of the validation sections. This would include the calibration range that is used to determine the linearity of the working range of standards that would be used in the method. The standards would need a linear relationship between the response and expected concentration for reliable results as the equation of the line is used to calculate the concentrations of the unknown samples. The calibration standard range would also be used for the calculation of the concentrations of quality control (QC) samples, which would be analysed in triplicate over 5 days to determine the bias and precision values (SWGTOX, 2013). These values calculate the closeness of the results to the true value and between each set of samples (Flanagan, Taylor, Watson, 2007). Precision can be separated into two sub-sections determining the within-run and between-run values, which looks at the variability of values within each day and over the length of the validation.

Limit of detection (LOD) and limit of quantitation (LOQ) are in line with the calibration range as both of these values determine the sensitivity of the instrument to the analytes using a specific method. These values represent the lowest standard that can be reproducibly detected on an instrument with a 3:1 (LOD) and 10:1 (LOQ) signal to noise ratio. Inaccuracies could arise in quantifications if drugs were carried over into other samples. Therefore, carryover and interference studies would be included to rule out the detection of drugs in subsequent samples. High concentration standards would be analysed then blank samples directly after to determine if drug carryover could be detected (SWGTOX, 2013).

Other factors could be investigated if relevant to the method, which includes drug stability and dilution integrity. Stability involves reproducing the normal storage and preparation conditions of the analytes at different concentrations. The processed sample experiment would require a number of triplicate samples to have undergone normal preparation, the first set to be analysed immediately then the remaining sets stored

under normal conditions and analysed at set times. The stability of samples is determined when the variation exceeds the acceptable bias values (SWGTOX, 2013).

From previous research, both model compounds and drugs have been used to determine the validity of methods. The diffusion profile of rhodamine B has been previously determined on the UV in relation to the movement through a venous wall (Skopp *et al.*, 1997). In addition, to make this research relevant to toxicology, drugs found in toxicological casework were also included. The antidepressants amitriptyline and the metabolite nortriptyline are frequently found in numerous cases in therapeutic and toxic doses (Statistics, 2013). These drugs are most commonly detected using HPLC-DAD in the toxicology laboratory as part of standard procedure in drug screening and quantification.

3.2 Aims

The aims of this chapter were to validate three drugs (rhodamine B, amitriptyline, and nortriptyline) on two instruments to allow for the determination of the drug concentrations in the bladder diffusion studies. Rhodamine B was validated on the UV-Visible spectrometer and the HPLC-DAD was used to validate amitriptyline and nortriptyline.

3.3 Method Development

Previous research has suggested that post-mortem diffusion could occur from the bladder (Moriya and Hashimoto, 2001) however, there has been no further investigation into the extent of diffusion over time. Previous work by Skopp and colleagues (Skopp *et al.*, 1997) used a xanthene dye (rhodamine B) as a model in a method that mimics passive diffusion through the wall of a vein. The methodology involved using Franz cells to allow contact of the dye with the tissue and sample over a period and measure the solution in the acceptor chamber with a UV-spectrometer. This research utilised the same methodology and model compound however, it is important to confirm the suitability of the methodology with thicker bladder samples and to validate that UV-Visible spectroscopy can measure the rhodamine B concentration accurately and precisely. In addition, a model drug that is considered to undergo PMR is amitriptyline and therefore was chosen as it has been previously used in research related to PMR with the investigation of post-mortem release of the drug from the lungs of rats (Hilberg, Mørland and Bjørneboe, 1994).

3.3.1 Measurement of Diffusion from Franz-Cells

The inter-bladder diffusion studies needed only one section of the bladder from six different bladders, the sectioning had to be standardised to allow the sample to be taken from the same area each time for consistency. As a result, the right side of the bladder was chosen, as this is the usual side for femoral vein sample collection at autopsy (to mimic real life events). The middle of the bladder was chosen to reduce the amount of variation in thickness within the same bladder tissue section as large differences in thickness could influence the diffusion rate. The method for obtaining the middle of the bladder was to measure the length of the bladder from top to bottom using the callipers (for accuracy) and halving this value so the middle of the donor chamber is located in approximately this area when the bladder section is cut from the rest of the bladder.

3.3.1.1 Franz Cell set-up – Initial Method

Franz cells have been used previously in another study to determine the diffusion of rhodamine B and morphine through a venous wall. The vessel was sectioned then secured onto the cell and the donor and acceptor chambers were filled with rhodamine B or spiked blood samples. This study was carried out over 120 hrs (Skopp *et al.*, 1997). This method was used as the basis for this set of diffusion experiments as this is the most suitable method for recording of direct diffusion through biological tissue.

3.3.1.2 Equilibration

To allow for equilibration of the bladder tissues to the experimental conditions they were set up on the chambers and left for 30-45 minutes, before the donor chamber solution was added, and the first sample was taken.

3.3.1.3 Sample replacement

Initially, all samples removed for analysis were replaced into the chambers, as removing the solution decreased the volume (and thus the concentration) of sample that has diffused into the acceptor chamber. This would result in the next sample being slightly less concentrated. However, this would not give sink conditions, which would be more suitable for this experiment as it is over numerous days. Sink conditions involve keeping the dissolution solution from saturation as this would reduce the amount of drug able to diffuse into the solution affecting the rate of diffusion and the results of the experiment. If the samples were to be continually replaced then at some point equilibrium will be established between the two solutions and the tissue. This would prevent any more diffusion of the drug into the acceptor solution, which would not be accurate in mimicking the conditions within the body. As any drug that has diffused out of the bladder into the body would continue to diffuse away from the bladder. However, this experiment holds the drug underneath the bladder, this restriction in drug movement results to resorting to another method for removing a proportion of the drug to allow for more diffusion. As a result, after sampling, the volume in the acceptor solution was replaced with blank PBS or AA.

3.3.1.4 Air bubbles

Pilot experimentation determined that bubbles were disrupting the diffusion. Initially, the acceptor chamber was filled with PBS solution and then the tissue was added. However, this made securing the tissue on the chamber difficult as the acceptor chamber solution made the tissue slippery and hard to handle. As a result, the bladder was mounted onto the chambers first, the donor chamber was secured, and then the acceptor chamber was filled with solution. Re-orientating the chamber (upside down) allowed the solution to be gradually introduced to the tissues and once the solution was in contact with the tissues it was not disturbed. This would reduce the production of air bubbles between the acceptor chamber solution and the tissue. Any air bubbles that did arise using this method, was eradicated by tilting the chamber until the air bubbles travelled up through the spout, and left the chamber. In addition, as adding the acceptor solution was the last step once the chambers were replaced into their holder the chambers were not moved again throughout the experiment. This was to minimise the chance of developing air bubbles in the acceptor chamber.

3.3.1.5 Tissue displacement

In the pilot experimentation, the tissue sank into the acceptor solution when setup in the Franz cell. Therefore, the middle of the tissue needed to be supported with a material that would not react with the tissue and still be able to allow for diffusion of substances through into the acceptor chamber. A steel mesh was used (8 mesh, 2 mm squares) that was placed beneath the tissue, then the Teflon (non-slip) ring on top of the tissue to keep it in place. The mesh needed to be smaller than the Teflon ring otherwise the Parafilm could not seal the opening between the donor and acceptor chamber and therefore the chambers leaked. The mesh was cut to size using the Teflon ring as a guide using wire clippers. The Franz cell usually secures membranes that are micrometres in thickness (Garland *et al.*, 2012) however, the thickness of the bladder tissue could range between 1-8 mm. The mesh was too thick alongside the Teflon ring and the full thickness of the bladder tissue. Over the period of the experiment, this could have damaged the bladder tissue. The mesh left indentations on the bladder tissue reducing the amount of diffusional area for the donor solution to pass through. In addition, as the thickness of the bladder was being investigated as a factor that could affect drug diffusion reducing the bladder thickness would give unrealistic results.

Alternative methods were tried to replace the Teflon rings, as these did not grip the bladder tissue, as it was wet and slippery. A non-stick reusable baking sheet was available and so rings were cut using the Teflon rings as a guide to see if this material would hold the bladder in place. Initially, the rings were cut the same as the Teflon rings (Fig 3.1), however the bladder needed to have more support so the diffusional area was reduced to support the bladder (Fig 3.2 and 3.3).

It was found that with two of the rings (See Figures 3.2 and 3.3) the bladder was distorted as the bladder was forced through the sections of the rings that were cut out. Therefore, bunching the bladder up into the acceptor chamber, this would increase the thickness of the bladder, which could not be measured. It was decided to use the original ring design (See Figure 3.1) and find another way to keep the bladders taut during the experiment. In addition, the surface area of the original ring design was larger with a value of 283.5 mm^2 in comparison with 188.50 mm^2 for Fig 3.2 and 129 mm^2 for Fig 3.3.

3.3.1.6 Paperclips

Initially, the bladders were placed onto the chambers by hand and if the tissue was not large enough to fit across the whole acceptor chamber the tissue was manually stretched and replaced onto the chambers. Alternatively, the tissue was mounted onto the chambers and secured with the clamp to stretch out the bladder. However, this was not sufficient to keep the bladder taut as in most cases the bladder would sag into the acceptor chamber or “bunch-up” into the donor chamber, which was usually wet and therefore slippery. In addition, the manual handling of the tissue, stretching and cleaning, could affect the integrity of the bladder membrane layers. As a result, a paperclip was employed to keep the bladder taut when setting up the Franz cell. Initially, a number of different orientations were tried to get the optimum method for keeping the bladder taut while securing onto the Franz cell. The successful method was to straighten the paperclip and mould into a “U-shape”. Then the bladder would be pierced at each bottom corner and the ends of the paperclip would then be pierced into the top corners of the bladder. This held the bladder taut and the paperclip would be

widened dependant on the bladder tissue size (See Figure 3.4). In addition, other reasons for improving this method included minimal handling of the tissue, as the urothelium is easily damaged and lost post-mortem (Jost, Gosling and Dixon, 1989). This was the main reason for using fresh bladders that were setup as close to time of death as possible.

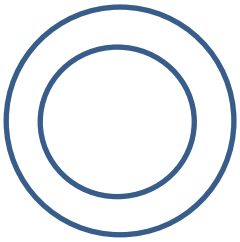

Figure 3.1 Original support ring design, cut from a reusable baking sheet to support the bladder in the Franz cell

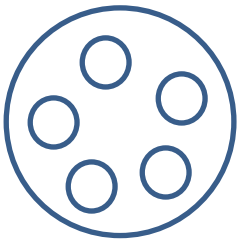

Figure 3.2 Circular ring support design, cut from a reusable baking sheet to support the bladder in the Franz cell

Figure 3.3 Linear ring support design, cut from a reusable baking sheet to support the bladder in the Franz cell

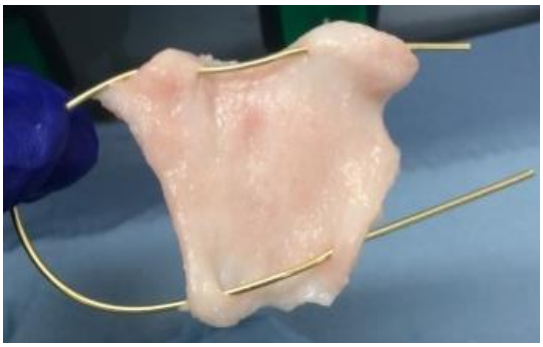

Figure 3.4 Image of a bladder tissue section secured onto the paperclip

3.3.1.7 System leakage

Initially, the chambers were secured with just a clamp and no Parafilm around the bladder, this was tried to see if the clamp itself was enough to prevent leakage from the bladders. This was not the case as there was a significant amount of leakage from the opening between the donor and acceptor chambers. As a result, Parafilm was placed around the outside of the chambers after the tissue was secured with the clamp, which reduced leaks for 2 days. However, leaks still occurred, the method of applying the Parafilm had to be improved. When started using the paperclips to keep the bladder taut the Parafilm was put around the bladder itself to prevent contact of the bladder with the outside environment as this dries out the bladder and could shrink allowing for an opening between the two chambers. The Parafilm was secured by piercing the ends of the expanded Parafilm on either ends of the paperclip. This did not improve the situation therefore it was discontinued. However, the Parafilm that was applied to the outside of the bladder was first placed within the clamp so the Parafilm sat flush with the opening between the two chambers. Then both ends were stretched around the opening between the two chambers, using two pieces of Parafilm for a better seal. This method showed a marked improvement, with no leakage from all of the chambers. This improvement of the method was then adopted within the setup method for the Franz cell.

3.3.1.8 Sample preparation (Sample Filtration)

Pilot experiments did not involve any sample preparation prior to analysis on the UV spectrophotometer. However, initial results showed higher readings than expected. A comparison of the absorbance values of the initial diffusion experiments, to the samples after filtering using cellulose acetate filters (Chromacol, 17 mm, 0.45 µm) resulted in a lower reading that was produced by filtering the samples (See Figure 3.5). This showed that most of the absorbance was due to degraded bladder tissue in the solution, especially at higher temperature the tissue degraded faster, resulting in more tissue in the solution and therefore higher absorbance.

The filtration experiment was carried out to determine which use of cellulose acetate filters had the best recovery of rhodamine B solution over 5 days (length of bladder studies). The parameters included analysing a sample with no filtration, single use of cellulose acetate filters and re-used cellulose acetate filters. This was to determine if the filters could be re-used to filter samples during the experiment. The 5 mg/L of rhodamine B solution was chosen to test the filters. Two samples of 40 ml (5 mg/L) rhodamine B solution were prepared from stock and put into two beakers, sealed with Parafilm and placed into an incubator (GallenkamÞ, size two) at 37°C. Sampling was carried out twice a day over 5 days, transferred 0.5 ml using a plastic Pasteur pipette into small glass vials for transfer to the UV-Visible spectrometer. A 1 ml syringe (1 ml Terumo syringe without needle) was used to remove 1 ml of the sample for directly filtering into the quartz cuvette. When using the reused filters, air was pushed through the filter before used on the next sample to reduce carryover of samples. When running the samples on the UV-Visible spectrometer each sample was run three times to get an average result. Two beakers of solution were used to get an average result.

In addition, the optimum wavelength (λ max) was determined for the rhodamine B at 554 nm when run on a scan. However, the value from the Skopp (Skopp *et al.*, 1997) paper was used for the experiments (543 nm)

a comparison was carried out to determine if using the 543 nm was detrimental to sensitivity in picking up and quantifying the drug in the samples. A calibration and QC set for the experiment described above were analysed at 543 nm and 554 nm to determine if there was a significant difference in the detection of the drug. The results showed there was no difference in sensitivity of the drug between 543 nm and 554 nm. The r^2 value for 543 nm using the calibration line 0.156 - 5 mg/L was slightly better than at 554 nm, r^2=0.9998 and r^2=0.9996 respectively. As a result, the initial wavelength continued to be used for the remainder of the rhodamine B based studies.

Samples that underwent no filtration had the highest results at 7.35 mg/L (See Fig 3.5) as the bladder tissue would have degraded into the solution causing it to be cloudy absorbing more light. Therefore falsely increasing the concentration of rhodamine B. Re-using the same cellulose acetate filter was successful for up to 12 samples; the same filter was used for both beakers, to remove any bladder tissue from the solutions containing rhodamine B. The highest concentration using the same filter was 6.8 mg/L and using a new filter each time had a maximum concentration of 5.3 mg/L. This had no effect to the drug itself (no retention in the filter) resulting in good recoveries of rhodamine B with an average recovery of 99.4%. Using a new filter each time worked for all samples, however this is not feasible so using the same filter as far as possible will be incorporated into the bladder studies method.

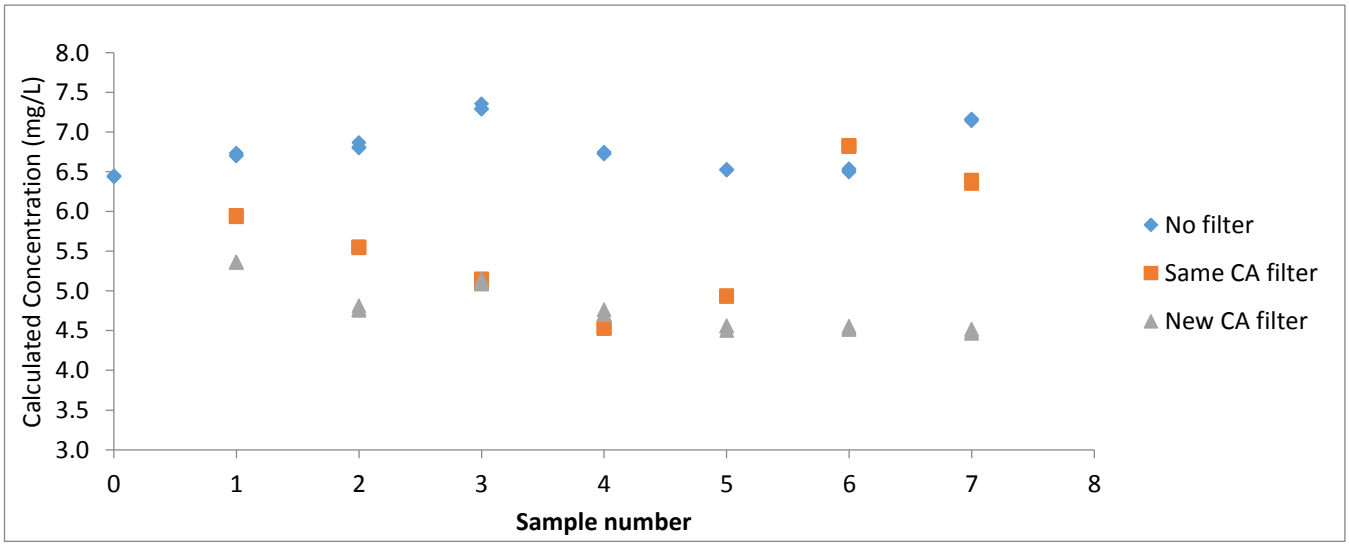

Figure 3.5 Comparison of 5 mg/L rhodamine B standards before and after filtering (n=2)

3.3.1.9 Sample volume

The sample volume was initially 4 ml to allow for filling the cuvette for analysis. However, this resulted in taking a larger volume that could be held in the spout. As a result, this would reduce the concentration as 27% of the total acceptor chamber volume would be removed per sample point and also create air bubbles in the acceptor chamber when returning the sample. As a result, the sample volume was reduced to 0.5 ml, which was the volume of the spout, to reduce the risk of introducing air bubbles during the sampling process and reducing the effect on concentration change.

3.3.1.10 UV Cuvette

In order to take into account the reduced sample volume the cuvette used in the pilot experimentation (plastic, (Fisherbrand Polystyrene Macro Cuvettes for Visible Wavelengths, 4ml) was replaced with a quartz, 400 µl volume, cuvette (Hellma Analytics, high precision cell, synthetic quartz light path 10 mm).

3.3.1.11 Franz Cell temperature stability

In order to determine the temperature within the Franz cell was constant and accurate an experiment was carried out to determine the temperature across all six chambers of the Franz cell. The set temperatures were 37°C, 20°C, and 5°C. The solution used was deionised water as this was the base of both the buffers used in the bladder diffusion studies. The method for this experiment included turning the water bath on to the appropriate temperature (37/20/5°C) and equilibrated for 2 hrs. Deionised water was added to all 6 chambers, the spout was sealed and the top of the acceptor chamber with parafilm to prevent evaporation. Also, to allow the water to equilibrate to the correct temperature for ½ hour. After equilibration, the thermometer (Fisherbrand Red Spirit Filled Partial Immersion Thermometer) was inserted into each chamber and the temperature was recorded. A number of samples were taken over the course of the five days. The magnetic stirrers were switched on for all chambers throughout the experiment, to mimic the bladder studies as closely as possible. The fume cupboard light was turned off between sampling to mimic bladder studies. The results for the three temperatures consisted of 144 readings over the 5 days per temperature resulting in consistent results. The 37°C experiment had a consistent experimental temperature of 36 ± 0°C, the 20°C experiment was 21 ± 0°C, and the 5°C experiment had an experimental temperature of 7 ± 0°C. Although all three temperatures were slightly different from expected they are all consistent and stable at these temperatures. This shows there was no temperature fluctuation throughout each day and between days for each experimental temperature. As a result, all three sets of bladder studies are at stable temperatures throughout the entire experiment.

3.3.2 UV Visible Spectrometer

3.3.2.2 Initial Method

In order to validate the method for the concentration determination of rhodamine B on the UV-Visible spectrometer for use on the Franz cell a fresh calibration line and QC samples were prepared in pH 7.4 and analysed on a UV-Visible spectrometer three times per day over 5 days for precision (within-run and between-run) and bias at 543nm. The calibration range covered 1.56 mg/L - 50 mg/L, in accordance with the Skopp method (Skopp *et al.*, 1997) using a serial dilution. The QC sample was the middle concentration (12.5 mg/L) from the calibration line, this was used instead of using the SWGTOX guidelines (SWGTOX, 2013) stating the use of more QC samples, as replicating the method by Skopp and colleagues (Skopp *et al.*, 1997).

All values were corrected for the PBS blank solution. The results show the calibration graph using all six calibration standards for day 1-5 was not linear (See Fig 3.6) this was confirmed with a residual plot (See Fig 3.7), which shows saturation of the detector at the highest calibration standard (50 mg/L). As a result, the highest two calibration standards were removed from the graph to obtain a linear calibration line. The average r^2 value for the 1.56 mg/L – 12.5 mg/L calibration line was 0.9999 (See Fig 3.8) and the residual plot shows linearity (See Fig 3.9). The average QC samples were 15.5% away from the expected concentration. In conclusion, the calibrations were linear up to 12.5 mg/L with an average r^2 value of 0.9999 and the QC samples were all within the 20% bias margin. As a result, the calibration of 1.56 mg/L – 12.5 mg/L was used for the subsequent experimentation.

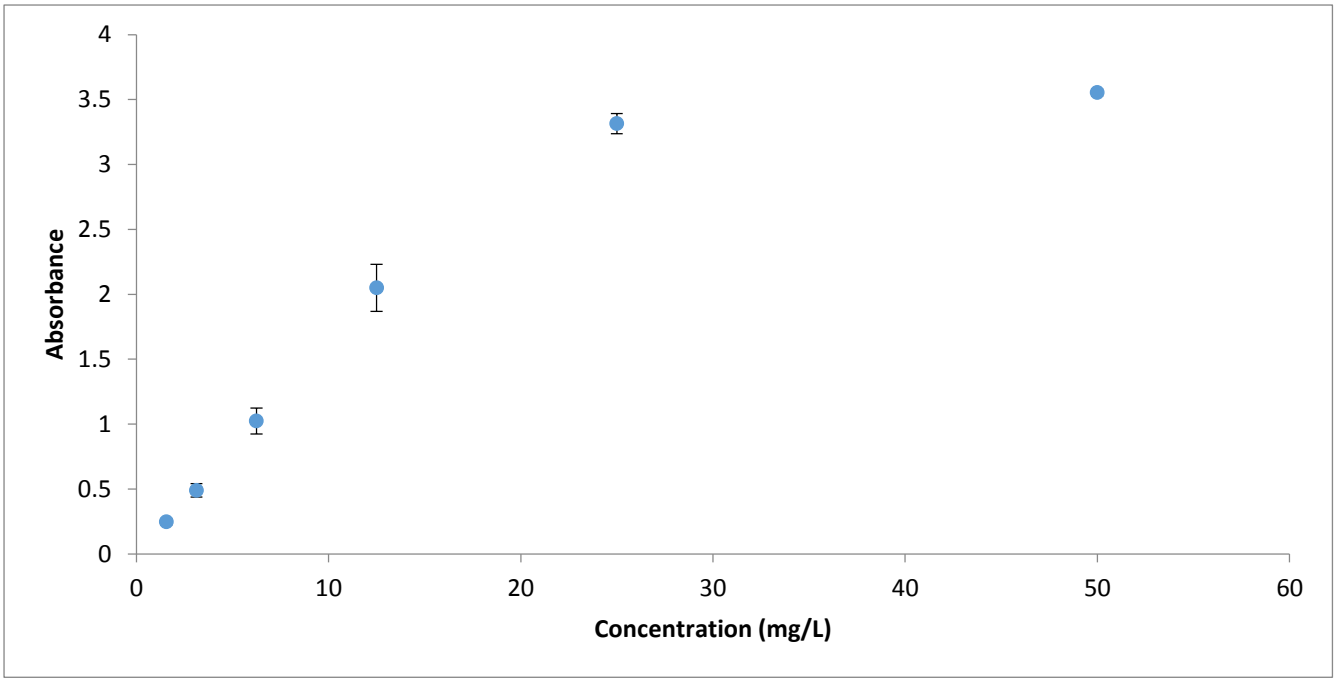

Figure 3.6 Average rhodamine B 1.56 mg/L - 50 mg/L calibration graph measured at 543 nm on UV-Visible spectrometer in PBS pH 7.4, (n=15) (Error bars are ± S.D.)

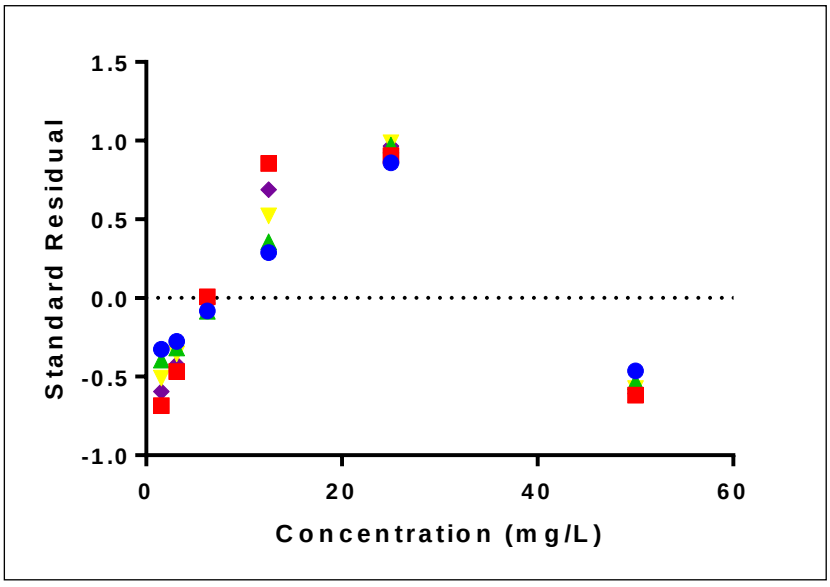

Figure 3.7 Residual plot for the calibration line of rhodamine B between 1.6 - 50 mg/L in PBS pH 7.4, (n=15)

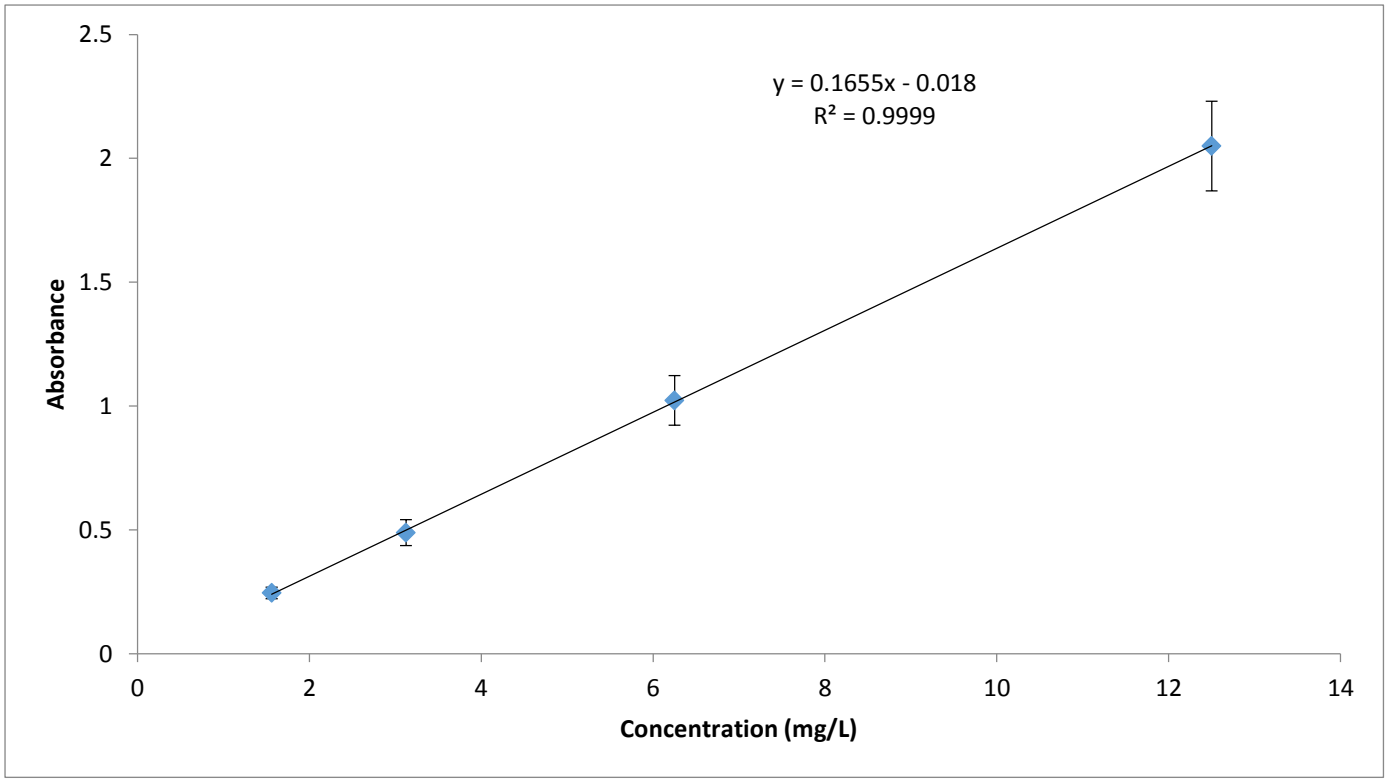

Figure 3.8 Average rhodamine B 1.56 mg/L-12.5 mg/L calibration graph measured at 543 nm on UV-Visible spectrometer in PBS pH 7.4, (n=15) (Error bars are ± S.D.)

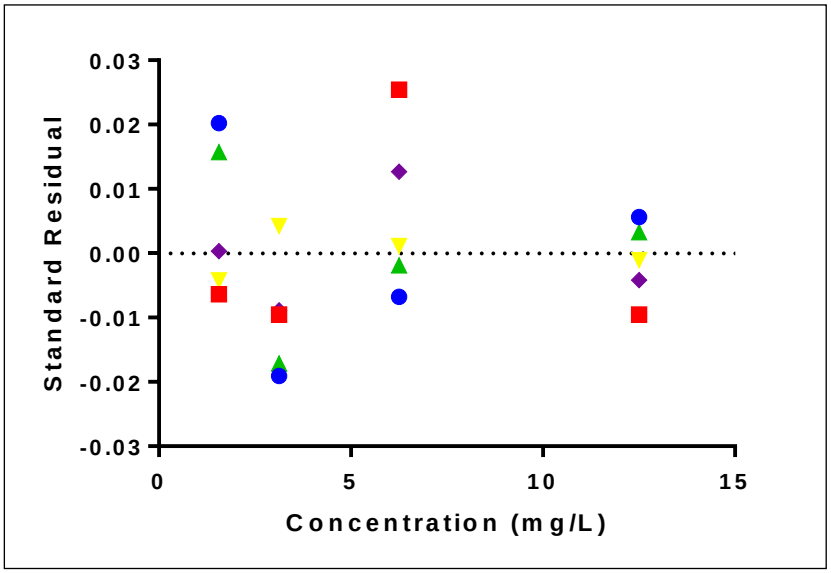

Figure 3.9 Residual plot for the calibration line of rhodamine B between 1.6 - 12.5 mg/L in PBS pH 7.4, (n=15)

3.3.3 HPLC-DAD

3.3.3.1 pH 7.4

3.3.3.1.1 HPLC set-up – Initial Method

The aim of this method was to identify the two drugs (amitriptyline and nortriptyline) with good peak separation (resolution), where the peak reaches the baseline before the second peak begins, within a short analysis time per sample (run time), ideally less than 10 minutes per sample. The starting point for the method development was taken from an in-house method from Centre of Forensic and Legal Medicine, University of Dundee. Drug stock solutions (1000mg/L) were prepared in water for amitriptyline and nortriptyline. Diluted standards of 10 mg/L were prepared for each analyte (amitriptyline and nortriptyline) and also a mixed standard of all two analytes at 10mg/L was prepared and analysed on the HPLC.

Dundee HPLC method: Column details: Waters Spherisorb 5 µm OD/CN, 4.6 x 150 mm Analytical Cartridge, column temperature 25°C. Isocratic mobile phase 57% organic (70% acetonitrile, 27.5% deionised water, 2.5% TEAP) and 43% aqueous (97.5% deionised water and 2.5% TEAP). Flow rate 2 ml/min, injection volume was 40 µl with a 7 minute run time at wavelength 210 nm.

All three drugs could easily be seen and identified in water (See Figure 3.10). The metabolite (nortriptyline) had a retention time of 3.0 minutes; amitriptyline was the next peak at 3.6 minutes . However, there was some peak tailing using this method.

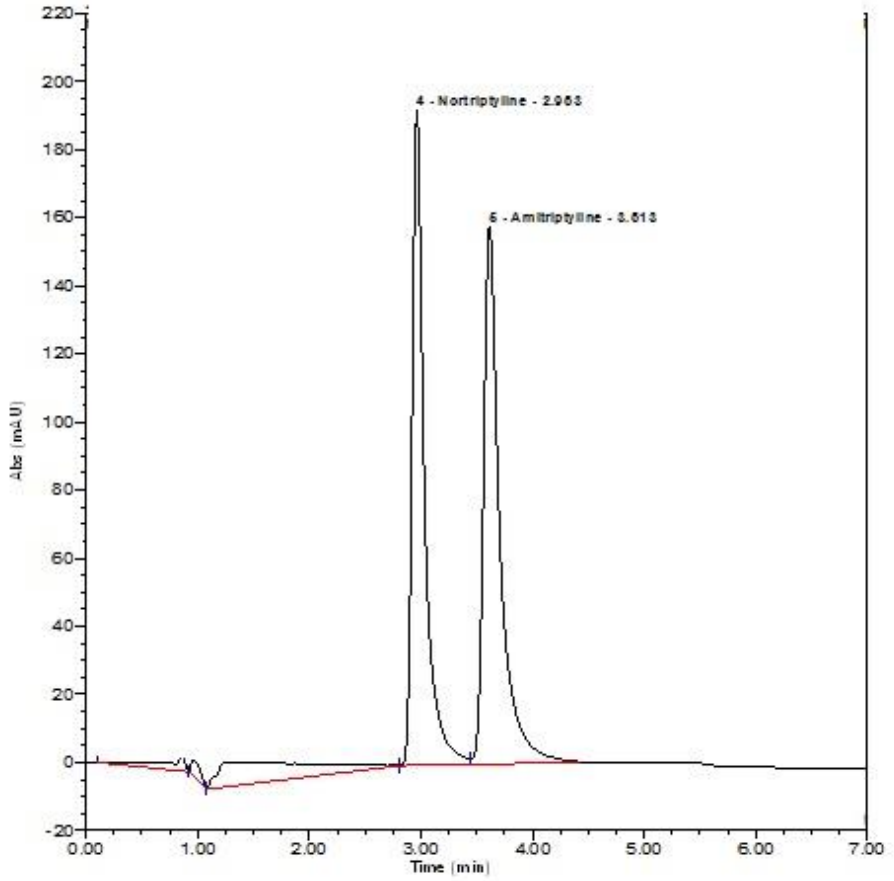

Figure 3.10 10 mg/L mixed standard of amitriptyline and nortriptyline dissolved in water analysed using the initial HPLC method

3.3.3.1.2 Method development – Peak tailing

In order to reduce the peak tailing to an acceptable level, dissolution solutions for nortriptyline were compared, with methanol and water to determine the most appropriate solvent. The solvent comparison was carried out for only nortriptyline as the peak in the chromatogram was merging with amitriptyline. The ideal asymmetry value is below 1.5 (Flanagan, Taylor, Watson, 2007). There was less asymmetry (Table 3.1) in water so nortriptyline was dissolved in water for all subsequent experiments.

Table 3.1 Asymmetry comparison of nortriptyline in two separate solvents

Solvent (Nortriptyline)	Asymmetry
Methanol	1.81
Water	1.77

In an attempt to reduce the peak tailing two, methods were compared with different isocratic mobile phase compositions. The first was 57% organic 43% aqueous and the second was 60% organic 40% aqueous. There was slightly less peak tailing at 57% A so used this method for subsequent experiments. However, there was still peak tailing due to the asymmetry values (See Table 3.2).

Table 3.2 Method comparison peak asymmetry

% Organic	Asymmetry
57 (Amitriptyline)	1.89
57 (Nortriptyline)	1.77
60 (Amitriptyline)	1.94
60 (Nortriptyline)	1.79

Furthermore, individual parameters were changed in the method to determine if the peak shape could be improved. Three parameters were changed that included reducing the injection volume to 30 µl, increasing the column temperature to 30°C, and increasing the TEAP buffer from 2.5% to 5%. Only the 10mg/L mixed standard sample was run for these method changes.

Table 3.3 Comparison of the asymmetry and resolution of both drug peaks in mixed standard samples for the HPLC parameter changes

Experiment / drug	Asymmetry	Resolution
amitriptyline:		
Initial Method	1.94	3.99
Reduced to 30 µl injection	1.91	4.02
Increased column temp to 30°C	1.68	2.85
Increased TEAP buffer to 5% in eluent	1.82	3.61
nortriptyline:		
Initial Method	1.86	3.21
Reduced to 30 µl injection	1.80	3.23
Increased column temp to 30°C	1.74	3.62
Increased TEAP buffer to 5% in eluent	1.74	2.84

Reducing the injection volume to 30 µl to reduce the peak merging between amitriptyline and nortriptyline did not affect the asymmetry of the peaks (See Table 3.3). The increase in the column temperature did reduce the peak tailing of the amitriptyline peak but did not affect the nortriptyline peak (See Table 3.3), however this is not a viable option as the buffer in the eluent is optimum at 25°C. If the temperature changed it may affect the buffer stability and as a result the pH of the eluent. Finally, the increase in buffer concentration, which was changed to improve pH stability, did not affect the asymmetry of resolution of the peaks (See Table 3.3). As a result, these changes were not implemented into the method.

3.3.3.1.3 Initial calibration analysis

In order to determine if peak resolution and asymmetry were similar across the calibration range a set of all samples (0.31 – 10 mg/L) was analysed once. As can be seen in Table 3.4, the resolution was above the threshold of 1.5, however there was still peak tailing. As this was an older column a new column was used to determine if the peak tailing was inherent in the methodology or due to possible column degradation.

Table 3.4 Comparison of asymmetry and resolution parameters between a set of calibration standards and QC samples

Sample / drug	Asymmetry	Resolution
amitriptyline:		
0.3125 mg/L	1.72	3.78
2.5 mg/L	1.76	3.79
5 mg/L	1.86	3.81
10 mg/L	1.91	3.76
LQC (0.5 mg/L)	1.74	3.83
HQC (5 mg/L)	1.83	3.80
nortriptyline:		
0.3125 mg/L	1.70	3.10
2.5 mg/L	1.78	3.10
5 mg/L	1.83	3.07
10 mg/L	1.87	3.04
LQC (0.5 mg/L)	1.82	3.13
HQC (5 mg/L)	1.79	3.09

3.3.3.1.4 The influence of a fresh column on peak tailing

In order to determine if the column rather than the method could be the cause of the peak tailing, a new column and a set of calibration standards (0.156 mg/L – 10 mg/L) and QC samples (0.5 mg/L and 5 mg/L) were analysed. The resolution (4.17 and 3.23) improved along with the asymmetry (1.32, 1.25 and 1.29) (See Figure 3.11) for amitriptyline and nortriptyline respectively. This showed that the original column was degraded resulting in the peak tailing. As a result, the replacement column (Waters Spherisorb 5 µm OD/CN, 4.6 x 150 mm Analytical Cartridge) solved the peak tailing issues.

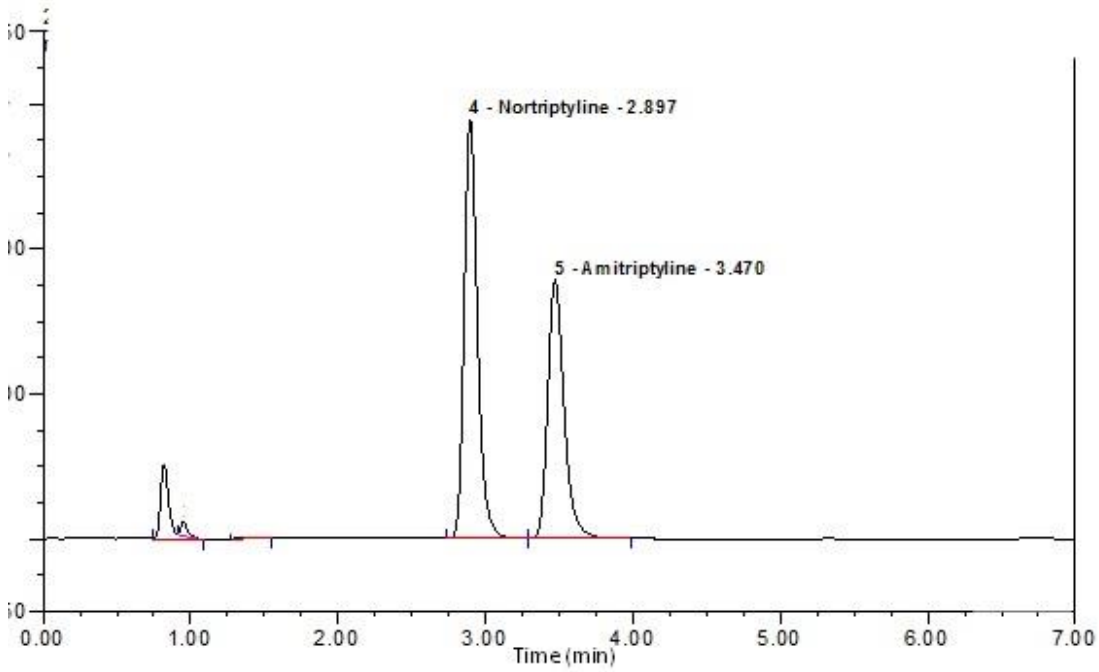

Figure 3.11 HPLC method development, pH 7.4: 10mg/L mixed standard (amitriptyline and nortriptyline) using the new HPLC column

3.3.3.2 Method development of amitriptyline and nortriptyline on the HPLC-DAD at pH 5

As the experimental plan required analysing samples at pH 5 it was important to develop a method for these samples.

3.3.3.2.1 Method development

Solubility of compounds can be a problem when using buffers in solvents and if not checked there could be precipitation of salts when the mobile phase is mixed in the HPLC, resulting in possible damage to the HPLC column and thus potential inaccurate results. The solubility of ammonium acetate (AA) (Sigma, BioXtra, ≥ 98%, Molecular weight: 77.08 g/mol) was mixed at various concentrations in acetonitrile as previous work has shown that AA may not be soluble in acetonitrile (Dolan, 2009). All 9 samples (90% - 10% acetonitrile to AA pH 5) were prepared therefore even if there was no buffer present in the organic phase the acetonitrile will be exposed to AA in the samples. The damage to the HPLC column from the salt precipitation would need to be minimised. The percentage of acetonitrile where the buffer would not precipitate out needed to be determined in case the buffer needed to be added to the organic phase.

Initially, a gradient system was used for the method to isolate the retention times the drugs eluted from the column, which would allow for the calculation of the percentage of organic phase that would be needed to change to an isocratic method. An isocratic method would reduce the amount of solution used and therefore the run time. The initial mobile phase consisted of the organic phase (80% CH_3CN: 20% 20 mM AA pH 5) and the aqueous phase (20 mM AA pH 5 dissolved in deionised water, 0.77 g AA in 500 ml). Buffer was added to both phases to cancel out buffer effects and salt precipitation when the two phases are mixed.

To equilibrate the HPLC before sample analysis, the eluent was changed to the initial mobile phase and 2 ml/min flow rate, which was used from the previous method as a start point. The system was purged and left to equilibrate for 20 minutes.

Initial pH 5 HPLC method: Column details: Waters Spherisorb 5µm OD/CN, 4.6 x 150 mm Analytical Cartridge, column temperature 25°C. Gradient method, 100% – 0% organic (10mM AA in 80% CH_3CN (20% AA to 80% CH_3CN)) and aqueous (10 mM pH 5 in water). Flow rate 2 ml/min, injection volume was 40 µl with a 20 minute run time at wavelength 210 nm.

This method identified peaks with nortriptyline at 5.1 minutes and amitriptyline at 7.8 minutes (See Fig 3.12). The specific percentage for the isocratic method was identified to be 59% aqueous 41% organic, which was tested with individual drug standards and a mixed standard of all drugs with a run time of 10 minutes. The run time was increased as no drugs were present. However even with the increase in time there were poor results from this method, which could be from too high a percentage of the aqueous phase.

If the pH is low i.e. pH.5 then basic drugs would be very ionised, both drugs have a pKa of 9, so they would not bind to the column if there was a higher level of the aqueous phase. Initially, no amitriptyline was detected from this isocratic method. Amitriptyline peaks were broad, the nortriptyline peak had inconsistent retention

times. The large difference in results between the two drugs of similar structure results in this method not being suitable.

The method development process at this point mainly only detected nortriptyline, with the initial gradient method showing amitriptyline. An isocratic method is still preferred as it would reduce eluent and run time usage. The isocratic method identified nortriptyline however, the peak shape was very broad. The run time for nortriptyline was very long. This isocratic method was too long which shows that there is either not enough organic phase to elute the drugs within a sensible run time or the column temperature could be too low resulting in reduced sensitivity for the drugs.

3.3.3.2.2 Revised method to quantify drugs at pH 5

Initial work looked at the suitability of the Waters column (Waters Spherisorb 5µm OD/CN, 4.6 x 150 mm Analytical Cartridge) for pH 5 analysis with the solubility of ammonium acetate, gradient method and isocratic method that was investigated but the column was found to be unsuitable. The Phenomenex column (Phenomenex Gemini 3µm $C_{18,}$ 110Å, 150 x 4.6 mm column) was found to be stable across pH 1-12 so was investigated as an alternative. A pre-used method was suggested from the Phenomenex applications to identify two drugs (amitriptyline and nortriptyline). Even with the wide range of stability, for both amitriptyline and nortriptyline to be neutral the mobile phase would need to be above pH 12 and would strip the column. Therefore, the suggested method was to use pH 4 - 5 that would cover the free silanols on the C_{18} column.

Method: Column: Phenomenex Gemini 3µm $C_{18,}$ 110Å, 150 x 4.6 mm column, column temperature was 30°C. Isocratic method with 30% aqueous phase (20 mM ammonium acetate dissolved in deionised water) and 70% organic phase (50% acetonitrile: 50% methanol). Flow rate 1.25 ml/min, injection volume was 40µl with a 20 minute run time at wavelength 210 nm.

The pKa of ammonium acetate is 4.8 so the working range was pH 3.8 - 5.8. The higher pH 5 was chosen to match the pH that was used in the bladder diffusion studies. Both mobile phases were filtered (Phenomenex, Phenex filter membranes, 0.45 µm, 47 mm, nylon)**.** Initially the run time was 20 minutes to ensure all the drugs would be present within the same runtime based on the results of this analysis the runtime was reduced to 10 minutes as all the drugs eluted within this time scale.

This method was a success as both drugs were identified and quantified with good resolution and within a short run time using an isocratic method, which would save eluent as no equilibration, time was needed between samples. Therefore this method would be used for subsequent analysis. Both the LQC and HQC were within the 20% bias value and therefore future samples should be able to be validated using this method. Very sharp and resolved peaks (See Figure 3.13) with acceptable asymmetry factors of 1.20±0.04, 1.19±0.07 for amitriptyline and nortriptyline respectively. In addition, the resolution values are 1.80±0.91, 2.68±0.04 for amitriptyline and nortriptyline respectively above the ideal resolution value of >1.5 (Flanagan, Taylor, Watson, 2007).

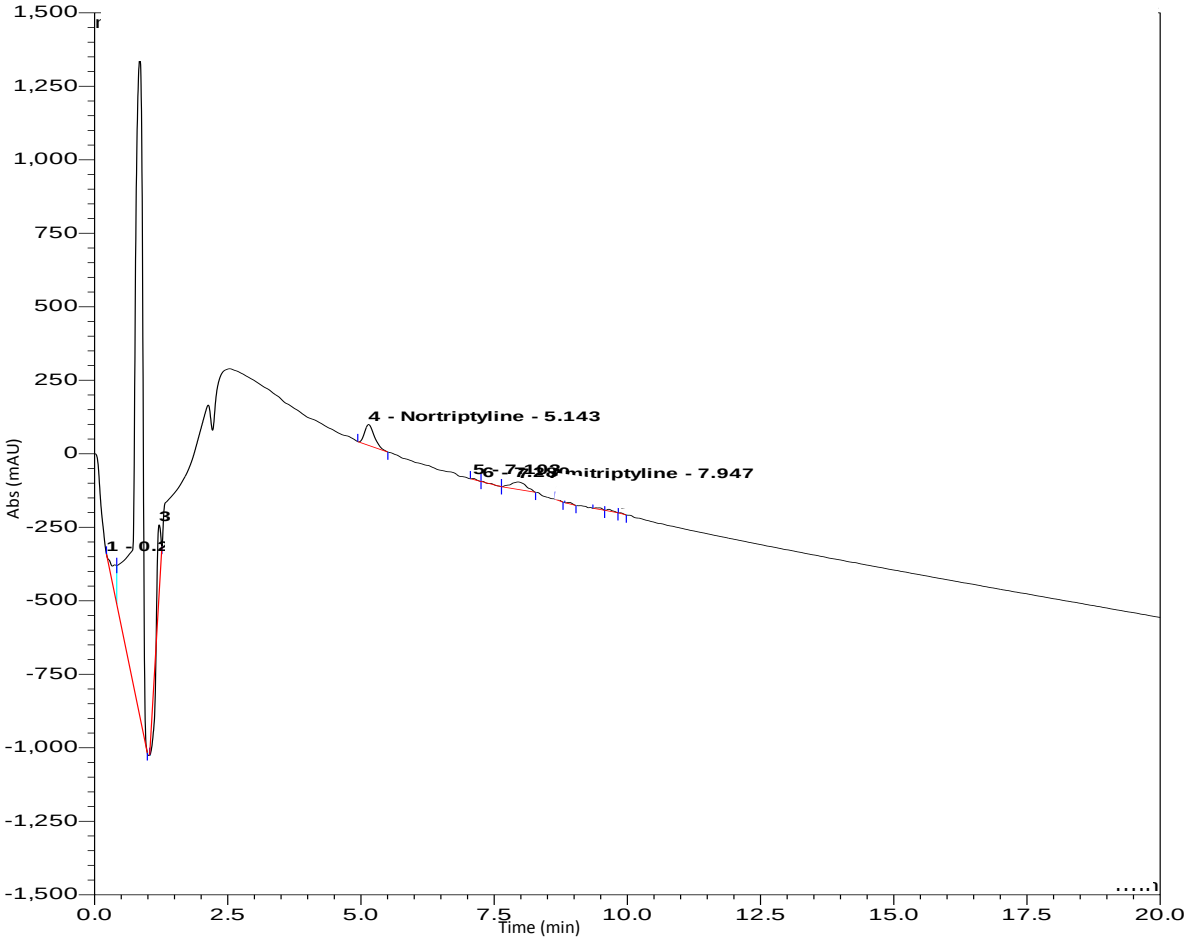

Figure 3.12 HPLC method development, pH 5, 10 mg/L Mixed standard (amitriptyline and nortriptyline), using the gradient method

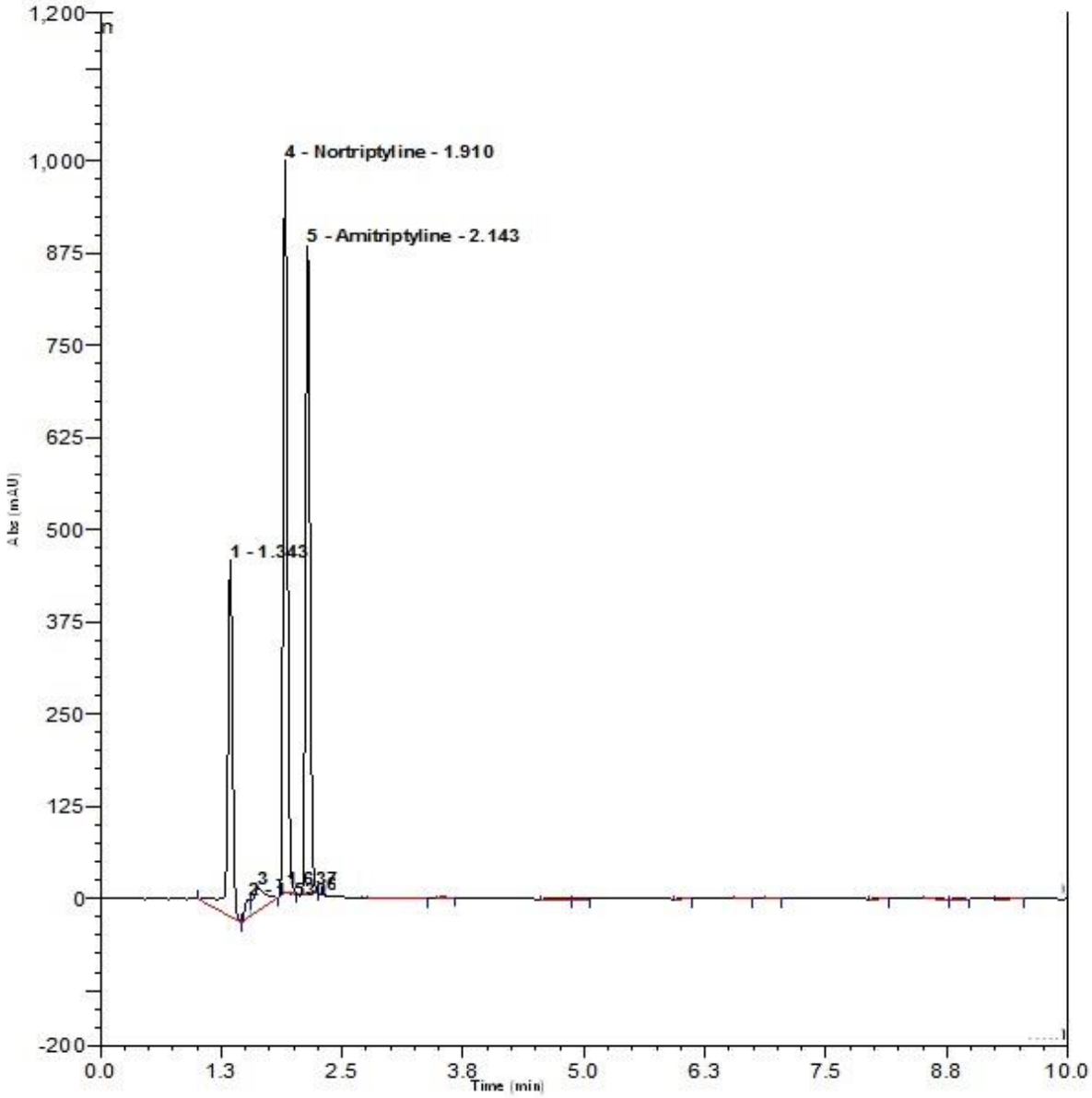

Figure 3.13 HPLC method development: pH 5, 10 mg/L mixed standard (amitriptyline and nortriptyline), using the suggested HPLC method

3.3.5 *In vivo* Rat Study

3.3.5.1 Catheterisation and preparation of the rat

An *in vivo* experimental model was used to determine if any bladder degradation occurred during a nine day period, if so would there be any diffusion from the bladder in that time and would it reach the femoral veins. The initial procedure to investigate was how to catheterise the rat. Previous studies were used in relation to the technique (Reis, Sopena, 2011) however, they were used on live rats and when the procedure was repeated on the dead rats there was difficulty in successful catheterisation. Therefore, as a guide, and to allow the catheterisation to be reproducible, an incision was made down the middle of the abdomen of the rat to visualise the organs, identify the bladder to observe the direction of the catheter until it had entered the bladder (See Fig 3.14). The bladder was initially filled with 100 mg/L rhodamine B as the pink coloured solution would be used as a visual indicator for when the bladder was full and to confirm there was no damage to the bladder caused by inserting the catheter (See Fig 3.15). The incision in the rat was closed with superglue and due to leakage of fluid from the urethra this was tied with cotton (See Fig 3.16).

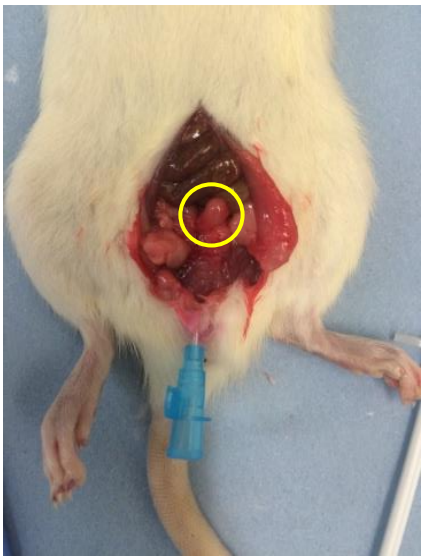

Figure 3.14 Midline incision into the rat exposing the organs to identify the bladder for catheterisation. The bladder is circled in yellow.

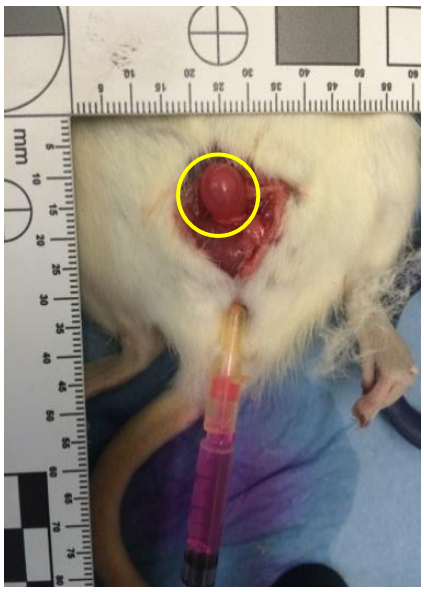

Figure 3.15 Exposed rat bladder filled with 100 mg/L rhodamine B solution to determine maximum volume. Bladder circled in yellow

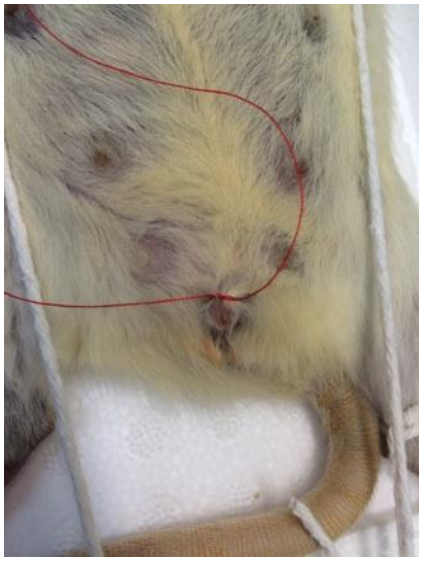

Figure 3.16 Rat urethra sealed with superglue and cotton string

3.3.5.2 Securing the rat for CT scanning

To allow the rat to be vertical in the Micro-CT the rat would have to be supported to stop movement during the duration of the study. Therefore previous studies involving CT scanning rats was researched and found a method using a polystyrene support and elastic bands (Judex *et al.*, 2010). It was found after approximately two days stood vertically in the fume cupboard sealed in the plastic weapons tube that the elastic bands alone would not support the rat . The weapons tube (Sourced from the University of Huddersfield Forensics department) is a two part plastic container that screws together. The elastic bands forced the organs down to the bottom of the peritoneal cavity and flattened the area around the band. This concluded the elastic bands were too tight and interfered with the rat. An alternative method was to use string to secure the rat to the polystyrene sheet in a way that would not push on the organs and move them for the correct anatomical place (See Fig 3.17). The support for the polystyrene sheet with the rat to remain vertical was found to be a plastic weapons tube as the polystyrene sheet and a medium sized rat would fit better than a large rat (See Fig 3.18). The plastic weapons tube was sealed using Parafilm. The polystyrene was cut to size to fit in the weapons tube (8.7cm diameter) using a hot wire cutter at 9 volts.

Figure 3.17 Rat secured to a polystyrene sheet using string and elastic bands

Figure 3.18 Rat secured to a polystyrene sheet and sealed in a plastic weapons tube for CT scanning

3.3.5.3 Determination of the CT visible drug to use in the rat bladder

The next part of the method was to determine the contrast agent suitable for the CT. The initial contrast agent used was sodium diatrizoate hydrate (Sigma, UK) as this is the compound that is found in the medical CT drugs including Urografin (Geenen, Kingma and van der Molen, 2013). The rat was catheterised and the bladder contained 0.33 ml of approximately 182.42 mg/ml of the compound, which was determined from the optimum iodine concentration of 0.27 M (Crespigny, 2008). The CT scan was started approximately 30 minutes after preparation of the rat. The resulting scan showed that the contrast agent could be visualised in the bladder, highlighting the organ (See Fig 3.19). However, this rat was stored at approximately 20°C for one week and then scanned again to determine if the contrast agent could still be visualised. No contrast agent was visible at the repeat scanning (No data shown). This is thought to be due to the short half-life of approximately 120 minutes (Geenen, Kingma and van der Molen, 2013). Due to the proposed study duration of 9 days an alternative contrast agent would need to be utilised.

Other materials were investigated including silver, which has been used in a number of CT based experiments with rats and in particular silver nitrate has been shown to be effective with a long half-life between 1-4 months (Lee and Kim, 2013). Therefore the rat was catheterised with 0.1M of the solution as a starting concentration. However, this concentration was not visible on the Micro-CT and so the concentration was increase to 1M and the process was repeated. This was successful and the bladder was highlighted (See Figure 3.20) resulting in this compound being used for the nine-day study.

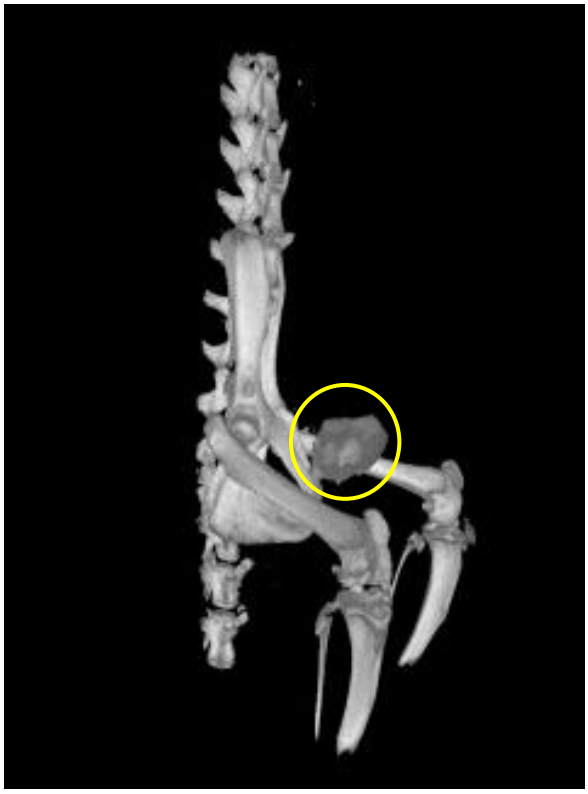

Figure 3.19 Micro-CT 3D rendered image of the rat skeleton and sodium diatrizoate hydrate in the bladder (circled in yellow)

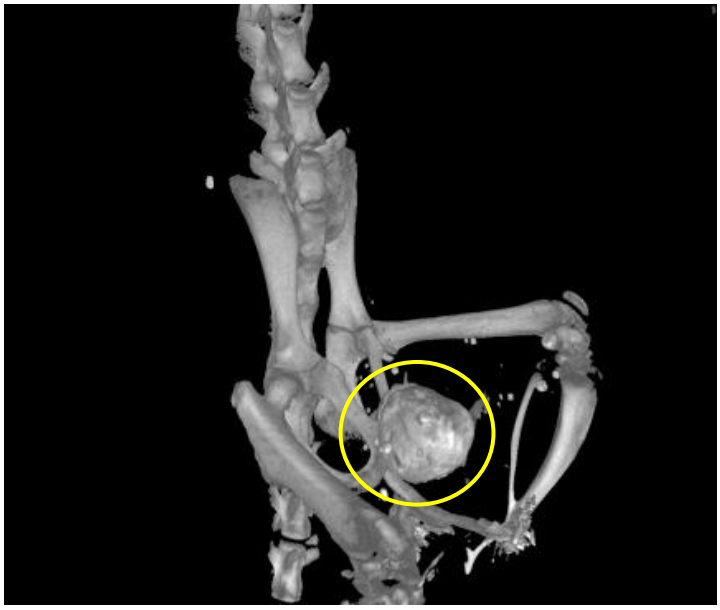

Figure 3.20 Micro-CT 3D rendered image of the rat skeleton and 1M silver nitrate in the bladder (circled in yellow)

3.3.5.4 Highlighting the femoral vein in the rat for the CT scan

As part of the aims of this section were to determine if any compound diffusing from the bladder could reach the femoral vein it was important to visualise the vessel on CT. It was not possible to locate or visualise the femoral veins using CT alone due to the lack of contrast between tissues (data not shown). Visualisation was attempted with the isolation, identification and then the marking of the femoral veins. In order to mark the femoral veins a substance that would be able to be visualised but not diffuse was needed. Therefore, a solid material would need to be secured to the femoral vein that could be visible on the CT. The preferred material was suggested to be aluminium and this was the base for fuse wire however, only alloy fuse wire was available. The method for isolating a femoral vein was found (Parasuraman and Raveendran, 2012) and utilised (See Fig 3.21), then using a microscope and a pair of tweezers the alloy fuse wire was tied onto the femoral vein using cotton string (See Fig 3.22). However, the CT scan with the fuse wire was not a success as the alloy reduced the resolution of the CT drug and the bone reducing both the to the same colour making them indistinguishable (See Fig 3.23). No measurements between the fuse wire and the bladder were made due to the reduction of resolution between the bladder and the bone. Thus it was decided that marking the femoral vein was not possible in this study.

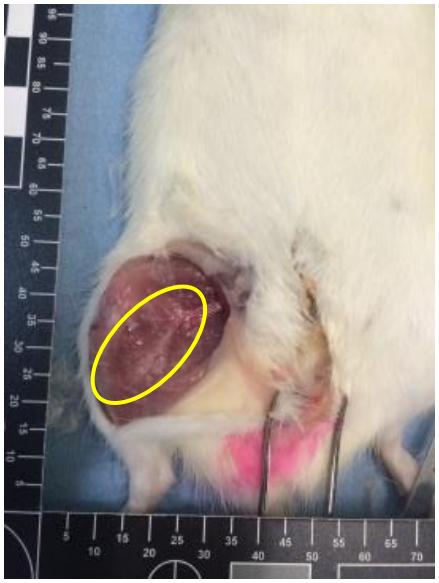

Figure 3.21 Identifying the femoral vein in the rat (circled in yellow)

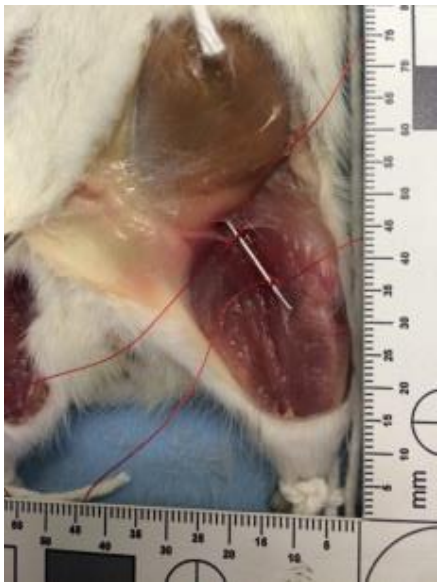

Figure 3.22 Alloy fuse wire tied to the rat femoral vein

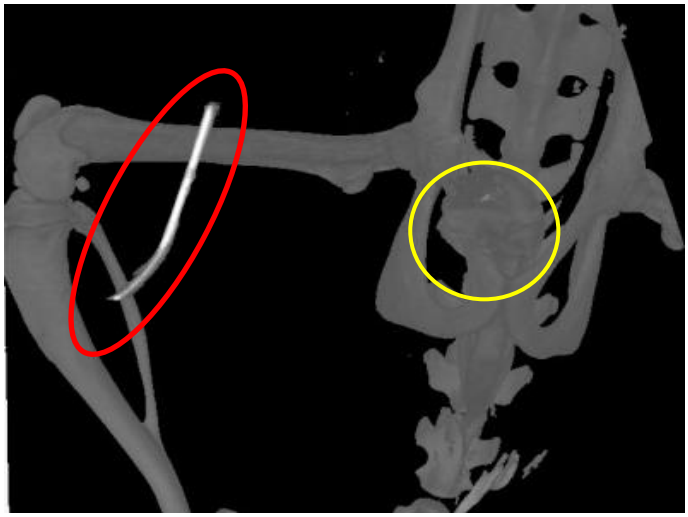

Figure 3.23 Micro-CT 3D rendered image of the rat skeleton, 1M silver nitrate in the bladder (circled in yellow) and the alloy fuse wire tied to the right femoral vein (circled in red).

3.4 Method Validation

Following the development of potentially suitable methods it is important to validate these for use. In order to ensure that the methods were fit for purpose, the quantitative methods of determining the concentration of rhodamine B (UV Visible Spectrometer) and amitriptyline and nortriptyline (HPLC-DAD) were validated according to the SWGTOX guidelines (SWGTOX, 2013).

3.4.1 UV Visible Spectrometer

For validation, it is important to state the required parameters as the experiments were meant to be quantitative therefore; a set of calibration standards (0.16 – 10 mg/L) and QC samples were required to validate the solutions on the instrument before running the experimental samples. A limit of detection and quantitation analysis determined the lower concentrations that were repeatedly detectable using the instrument. Two QC samples were used as prepared a serial dilution and therefore a linear relationship was expected. The two QC samples were 0.5 mg/L and 5 mg/L. To validate the solution on the UV a fresh calibration and set of QC samples were prepared from the same relevant same stock solution each day and compared using single factor ANOVA. The same stock solutions were used for all the calibration and QC samples to be able to also record the stability of the rhodamine B over the 5 day experiment. Initially, the calibration and QC samples were run three times a day to determine if there was any intra-day variation. The subsequent experiments consisted of calibration and QC samples run once a day to convert the absorbance of the experimental samples to concentration. In addition, dilution integrity samples were analysed to determine if the dilution of samples affected the resultant concentration. As during the diffusion studies some samples exceeded the calibration range preventing the calculations of the sample concentrations.

3.4.1.1 Linearity

The rhodamine B calibration standards were prepared as detailed in the methods (See Chapter 2 Section 2.1.5.1) to create a calibration curve containing 6 points covering a range of 0.16 – 5.0 mg/L. Five freshly prepared, separate calibration standards were analysed over 5 days. The absorbance was compared to the expected concentration to determine the linearity of the calibration range. As can be seen in Figure 3.24 and 3.26 both of the calibration curves of rhodamine B in PBS (pH 7.4) and AA (pH 5) had an r^2 of greater than 0.99. In addition, the residual plots for both pH solutions (See Fig 3.25 and 3.27) show the results follow the linear model resulting in acceptable linearity.

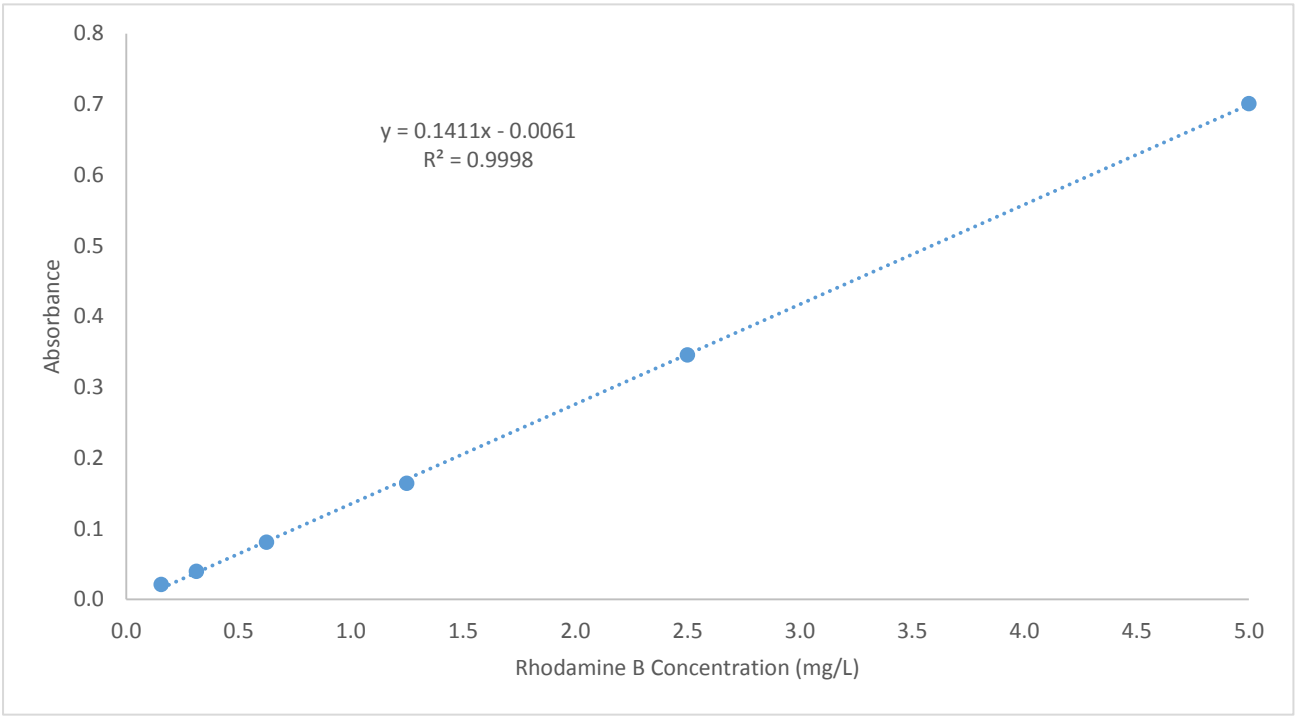

Figure 3.24 Average calibration graph for rhodamine B in pH 7.4 analysed on the UV-Visible Spectrometer.

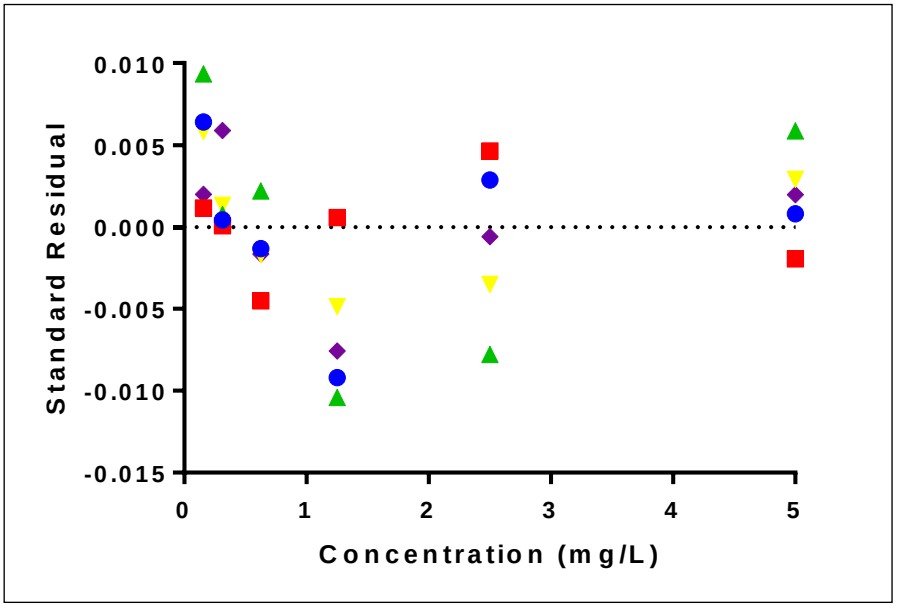

Figure 3.25 Residual plot for the calibration line of rhodamine B between 0.16 - 5 mg/L in PBS pH 7.4

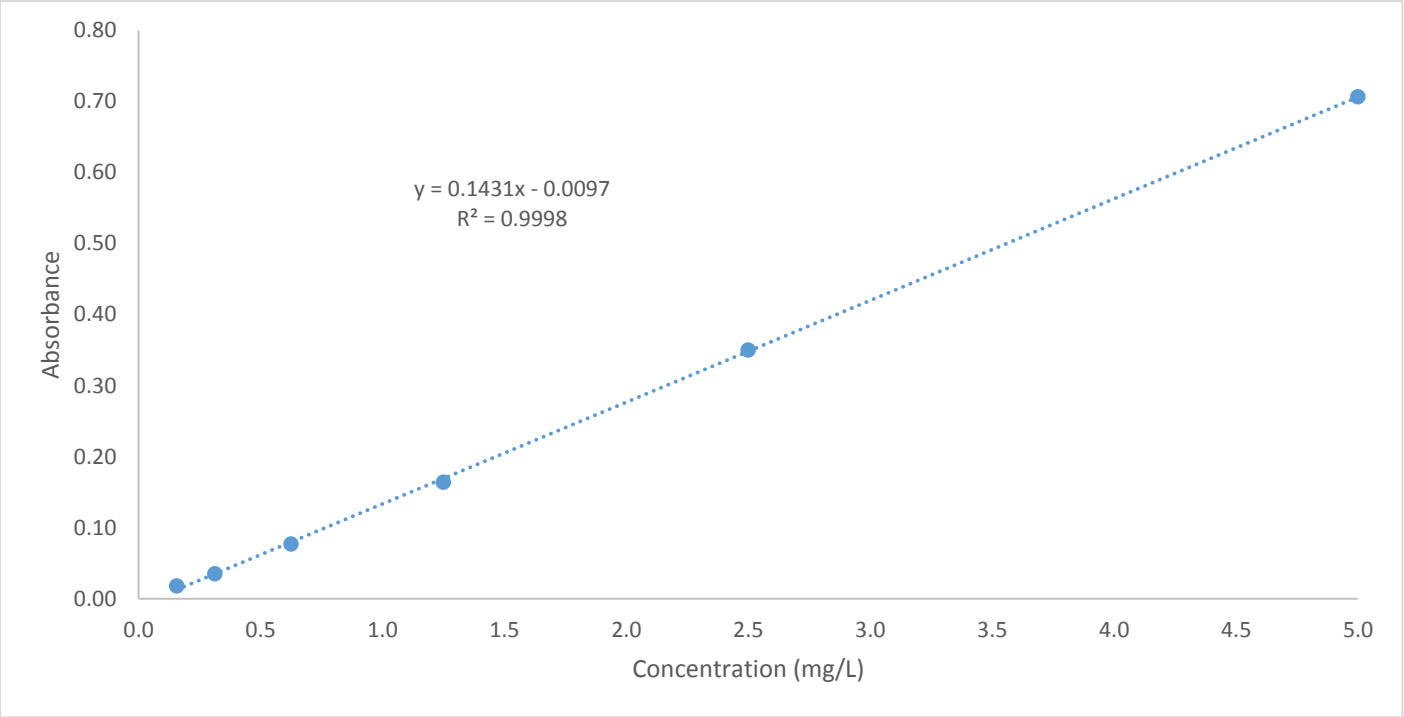

Figure 3.26 Average calibration graph for rhodamine B in pH 5 analysed on the UV-Visible Spectrometer.

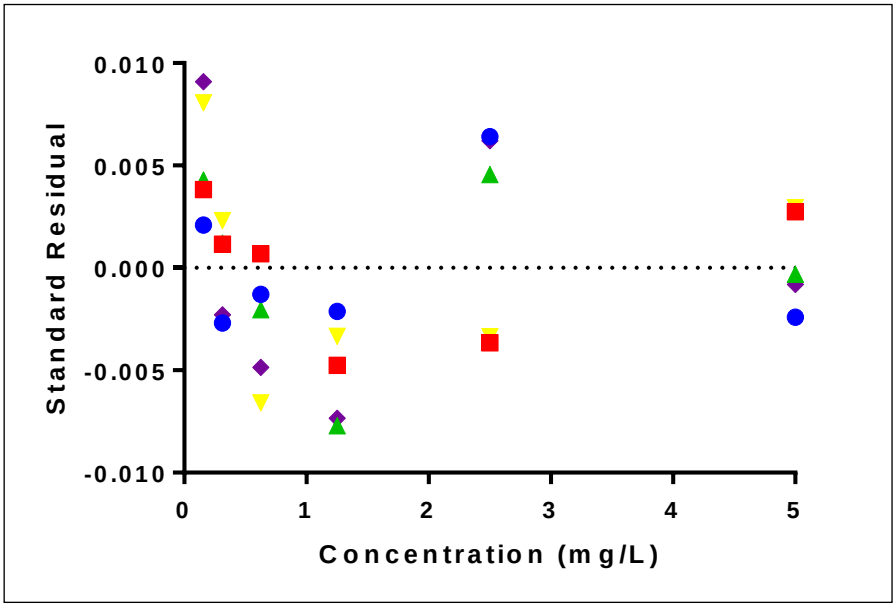

Figure 3.27 Residual plot for the calibration line of rhodamine B between 0.16 - 5 mg/L in PBS pH 7.4

3.4.1.2 Limit of Detection/Limit of Quantitation

In order to determine the LOD/LOQ three repeats of the lowest calibration standard were analysed over five days, the concentrations of the lowest standard were calculated using the equation of the line from the calibration graph. These concentrations were analysed in single factor ANOVA to see if there were reproducible results for within-run and between-run factors. The lowest calibrator passed the ANOVA with values < the 20% bias (See Table 3.5). Then the LOD and LOQ can be determined as the lowest calibrator (0.16 mg/L) for pH 7.4 and pH 5 for rhodamine B on the UV.

Table 3.5 ANOVA results for LOD/LOQ of rhodamine B in PBS and AA

ANOVA - 0.156 mg/L	PBS (pH 7.4)	AA (pH 5)
Within-Run (%)	6.81	3.74
Between-Run (%)	13.58	11.38

3.4.1.3 Precision and Accuracy (Bias)

Precision and bias were carried out to determine within-day and between-day variation over 5 days with three repeats of the quality control (QC) samples per analysis. The calibration standards were the same as described previously (Section 3.4.1.1), the two QC samples were determined to be 0.5 mg/L (LQC) and 5 mg/L (HQC). A separate rhodamine B 100 mg/L stock solution was prepared for the calibration standards and QC samples to mimic real life situations where the samples analysed alongside the calibration standards would originate from a different source. Single factor ANOVA was used to determine the precision and accuracy. As can be seen from Table 3.6 the precision and accuracy were <20 % and thus within acceptable ranges for further use.

Table 3.6 Intra-day and inter-day variation of rhodamine B in PBS and AA

	PBS pH 7.4		pH 5	
One Way ANOVA	**LQC (0.5 mg/L)**	**HQC (5 mg/L)**	**LQC (0.5 mg/L)**	**HQC (5 mg/L)**
Grand Mean (mg/L)	0.59	5.20	0.56	5.08
Bias (%)	19.70	4.17	12.28	1.76
Within-Run (%)	7.61	1.17	3.16	0.89
Between-Run (%)	8.43	1.64	6.38	1.54

3.4.1.4 Sample Stability

In cases where the samples may have to be stored or become unstable during the experiment before being analysed, it is important to determine if the samples are likely to suffer from instability. In order to investigate the stability fresh LQC (0.5 mg/L) and HQC (5 mg/L) rhodamine B samples were prepared from a separate 100 mg/L stock solution and analysed in triplicate. The samples were measured daily against a fresh calibration curve. The following variables were investigated including PBS (pH 7.4) at 4°C and 37°C, AA (pH 5) at 4°C and 37°C using a fridge (4°C) and a water bath (37°C).

The stability graphs for the LQC and HQC did not vary outside the recommended 20% bias (See Figure 3.28 and 3.29). This shows that rhodamine B was stable over 5 days in both solutions (PBS/AA) and both temperatures, fridge (4°C) and physiological (37°C). All five calibration and QC samples were run using the same stock solution and kept at ~4°C for 5 days. The results show a high r^2 value and the QC samples passing the ANOVA validation show that there is no significant variation between samples and so rhodamine B is stable in PBS and AA at approximately 4°C for 5 days.

Blank Solutions

Both blank PBS and AA solutions were analysed on the UV over 5 days, using the same solution, to determine if the blank solutions increase in absorbance over the course of the experiment. Duplicate samples of both solutions were stored in a humidity chamber at 37°C for 5 days and a sample analysed each day. The average absorbance value of blank PBS was -0.0042 ± 0.0022 and the average absorbance of AA was 0.0014 ± 0.0040. These low absorbance values result in no significant effect on samples during the experiment.

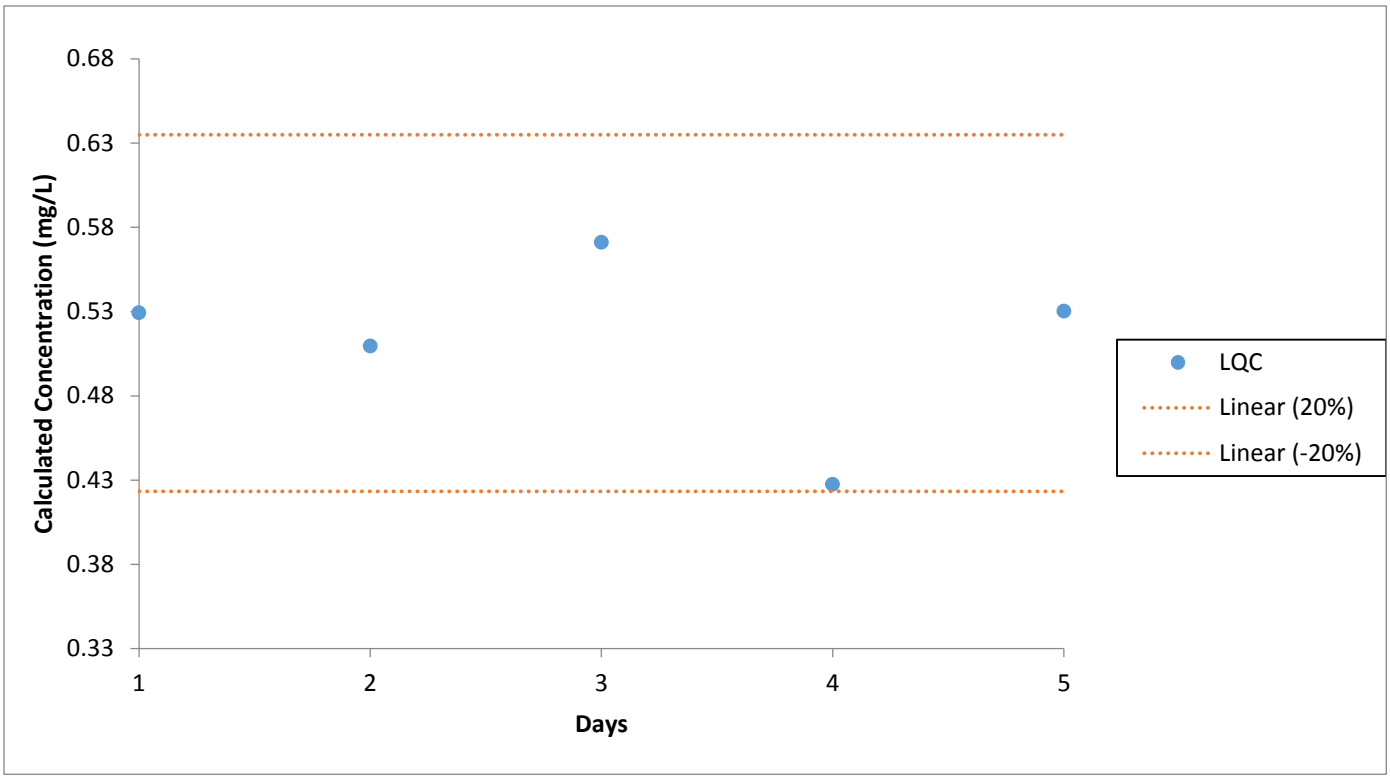

Figure 3.28 LQC (0.5 mg/L) 5 day stability of rhodamine B at 37°C in PBS pH 7.4 analysed on the UV

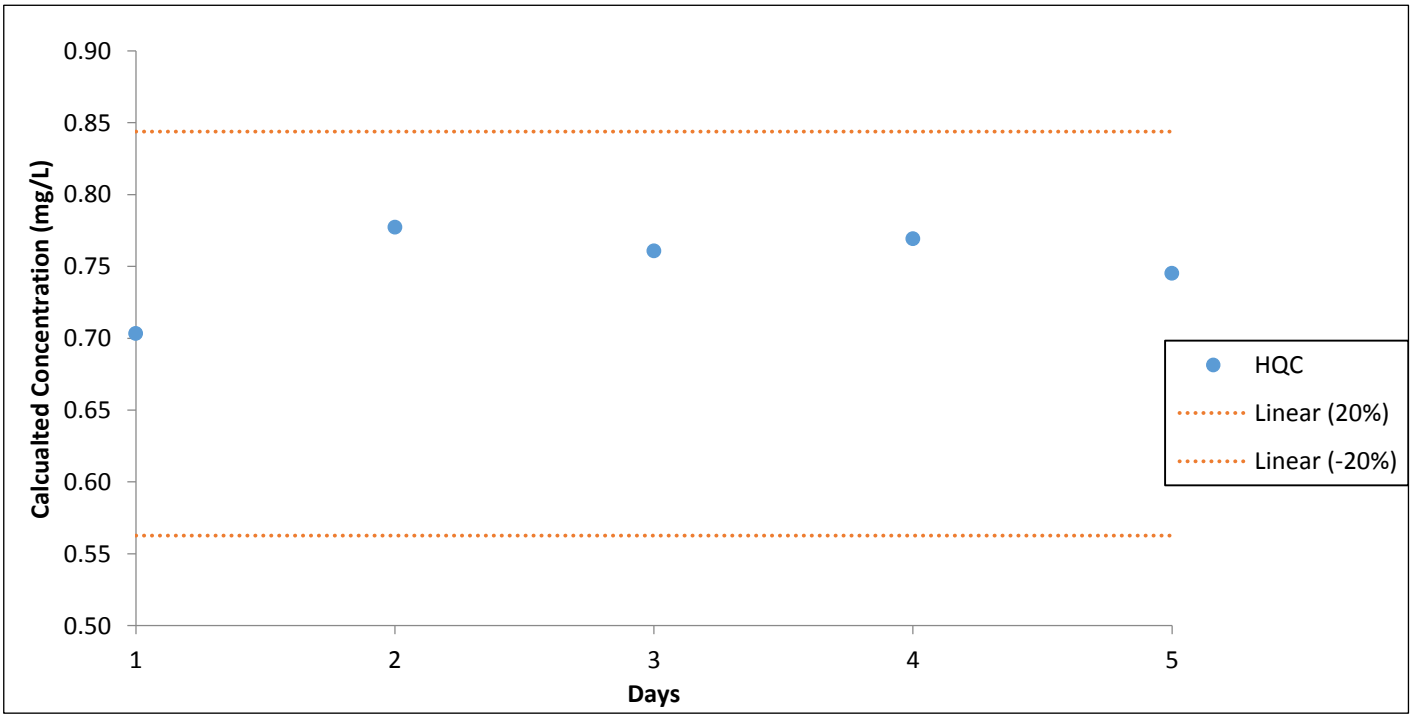

Figure 3.29 HQC (5 mg/L) 5 day stability of rhodamine B at 37°C in AA pH 5 analysed on the UV

3.4.1.4 Dilution Integrity

It is possible that concentrations in the diffusion experimentation may exceed the upper limit of quantification (ULOQ) of the calibration line and the sample may need to be diluted to be measured. In order to confirm that results of diluted samples are still accurate and precise. Various sample dilutions of 10 mg/L stock (1:1, 1:2, 1:10) and 100 mg/l stock (1:20) were measured. The average of the 3 repeats of each dilution factor calculated then converted from absorbance to concentration using the equation of the line of the calibration standards. The grand mean of each dilution factor and the bias was calculated. The concentrations were then analysed using ANOVA single factor to determine if the dilution factors pass the within-run and between-run factors (See Table 3.7). The results show that all four dilution factors passed the ANOVA and are able to be used in future bladder studies. As using these dilutions do not affect the resulting concentration, shown by the bias results and the ANOVA shows the reproducibility of the dilutions, which were all lower than the bias of 20% for both pH values.

Table 3.7 ANOVA Results for the dilution integrity samples of diluted 10 mg/L rhodamine B in PBS pH 7.4 using the UV-Visible spectrometer

	Dilution Factor – PBS pH 7.4				**Dilution Factor – pH 5**			
ANOVA	**1:1**	**1:2**	**1:10**	**1:20**	**1:1**	**1:2**	**1:10**	**1:20**
Grand Mean (mg/L)	8.87	9.32	9.23	103.74	9.39	9.86	9.39	104.67
Bias (%)	-12.12	-6.75	-7.67	3.74	-6.08	-1.37	-6.07	4.67
Within-Run (%)	3.03	2.05	6.00	1.08	4.05	2.66	7.40	1.72
Between-Run (%)	4.79	3.36	6.84	1.88	10.39	11.30	12.64	3.00

3.4.2 High-Pressure Liquid Chromatography with Diode Array Detector

The validation of the analysis of the drugs on the HPLC requires certain parameters to be included in the analysis. These parameters include the LOD and LOQ to determine if the drugs can be detected at acceptable levels that cover the expected calibration standard concentrations. The linearity is what determines if the set of calibration standards chosen (0.16 - 10 mg/L) can be detected and the relationship between concentration and absorbance is linear. The calibration range used matched the range chosen for the UV-visible spectrometer analysis. The precision and bias are the triplicate analysis of the concentrations of two QC samples that are determined from the equation of the calibration line. This analysis calculates repeatability of separate QC samples over 5 days. This analysis used single factor ANOVA (SWGTOX, 2013). The stability of the drugs was included in the validation as the drugs were stored over the course of the experiment and used on numerous days. In addition, the dilution integrity of drug samples was investigated as some samples during the course of the bladder studies were diluted to reduce the concentration to within the calibration line range. Finally, interference and carryover of drugs into blank solutions analysed directly after high standard drug samples would show if the column and method is suitable for the analysis of the analytes of interest.

3.4.2.1 Linearity

The linearity was validated to determine if the standards were linear between the highest and lowest calibration point. Then a line of best fit can be used to calculate an equation to determine the concentrations of samples compared against this line. The mixed calibration used standards of amitriptyline and nortriptyline . The range of 0.16-10 mg/L was prepared using a serial dilution. Five freshly prepared, separate calibration standards were analysed over 5 days and the UV absorbance from the HPLC-DAD to determine the linearity of the calibration range. The linearity results of both drugs in all solutions including deionised water, PBS and AA were > 0.99, showing linear results (See Figure 3.30 and 3.32). This is corroborated by the residual plots for the corresponding calibration lines (See Figure 3.31 and 3.33).

3.4.2.2 Limit of Detection/Limit of Quantitation

The LOD and LOQ were determined by analysing an extended calibration line 0.00015-10 mg/L to observe the lowest standard with a significant difference between the noise and the peak. The QC samples were kept the same at LQC (0.5 mg/L) and HQC (5 mg/L). The lowest standard where all three drugs in PBS and AA were present was 0.078 mg/L (See Figure 3.34 and 3.35); this is the standard below the current lowest calibrator (0.156 mg/L). This standard was included into an extended calibration graph, 0.078 – 10 mg/L to see the effect on the r^2 value. The r^2 value for nortriptyline and amitriptyline is 0.996 and 0.9962 respectively in PBS. The value for nortriptyline and amitriptyline calibration graph (r^2) is 0.9955 and 0.9981 respectively in AA. Adding this standard does not affect the linearity of the calibration line. However, this was not included in the final calibration as the initial calibration 0.16-10 mg/L had a higher r^2 value.

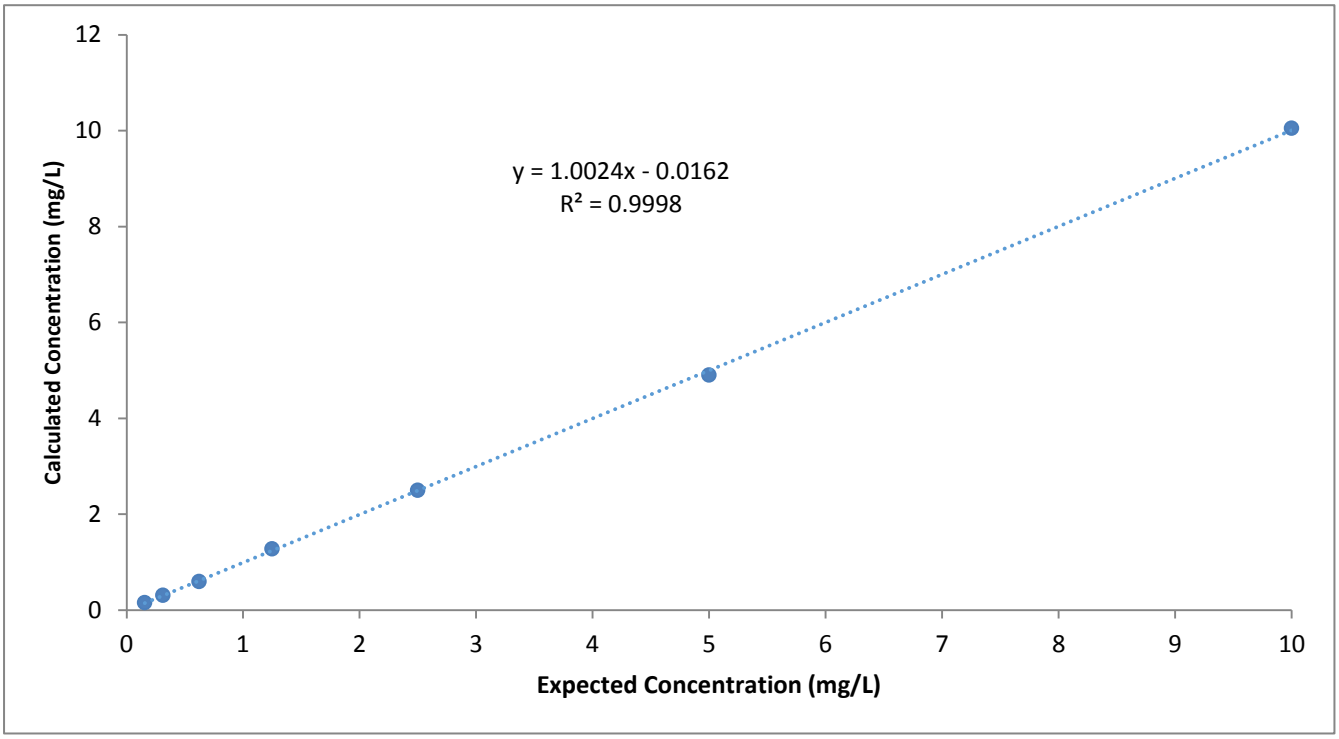

Figure 3.30 Average calibration graph for amitriptyline in pH 7.4 analysed on the HPLC

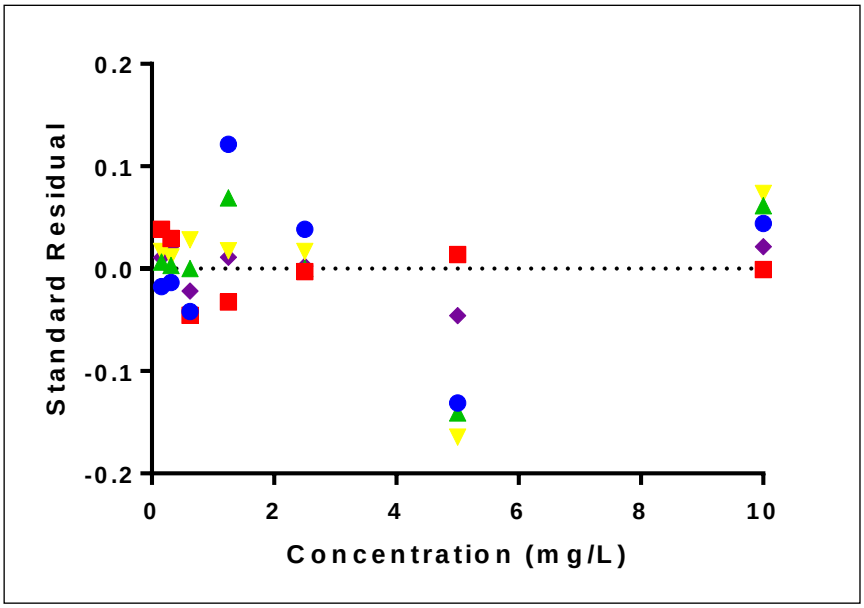

Figure 3.31 Residual plot for the calibration line of amitriptyline between 0.16 - 10 mg/L in PBS pH 7.4

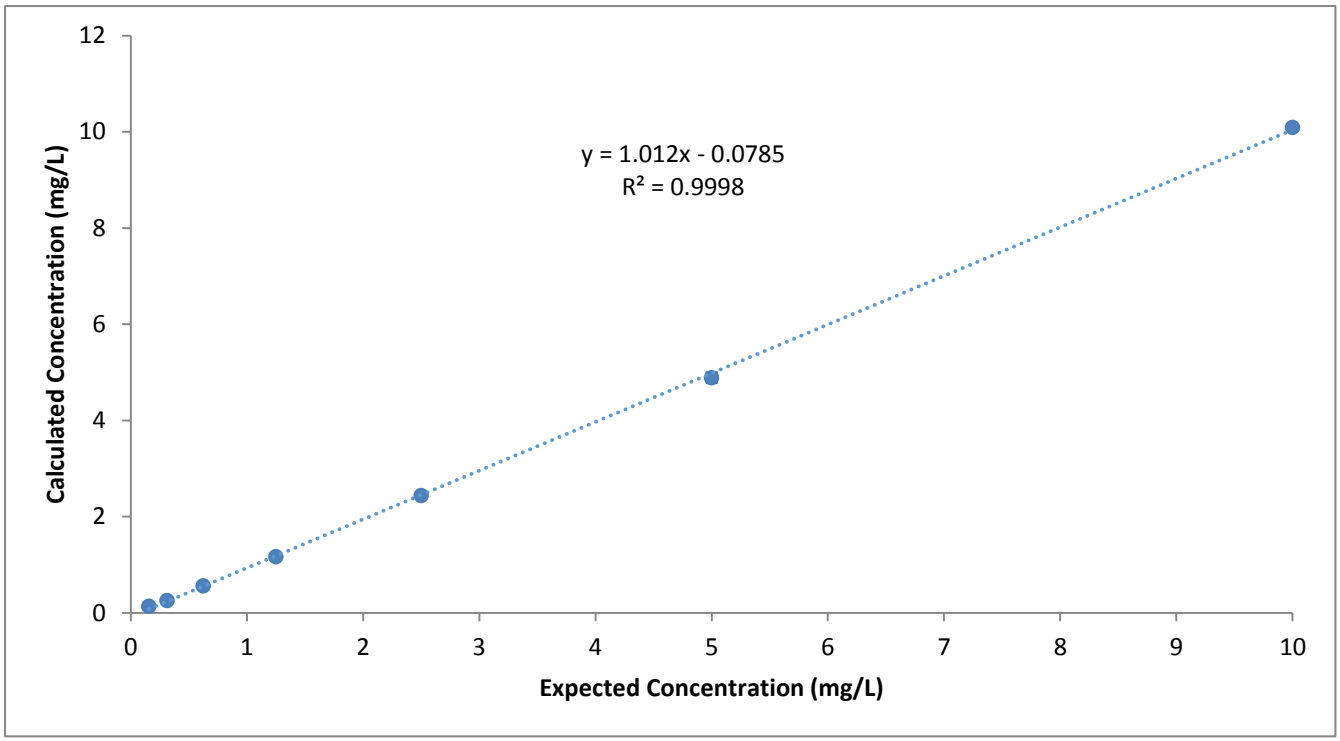

Figure 3.32 Average calibration graph for nortriptyline in pH 5 analysed on the HPLC

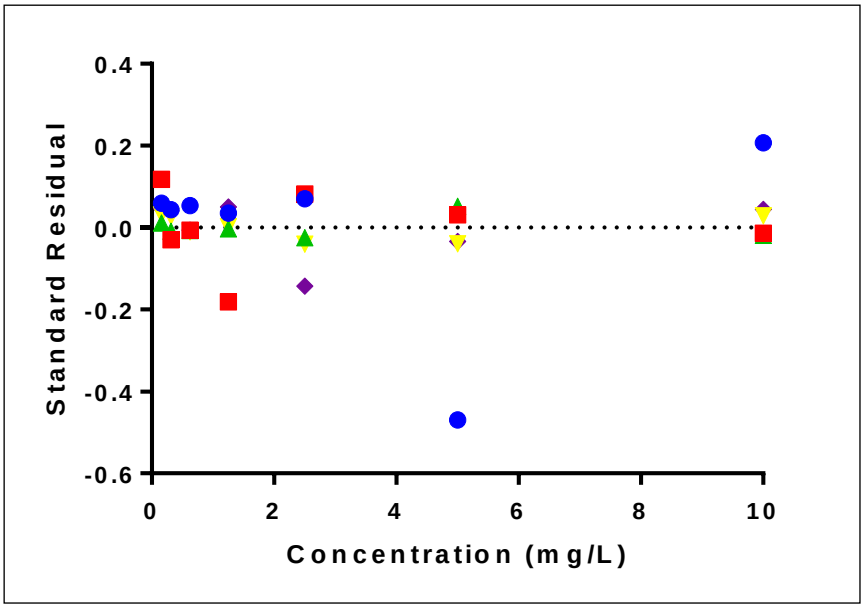

Figure 3.33 Residual plot for the calibration line of nortriptyline between 0.16 - 10 mg/L (pH 5)

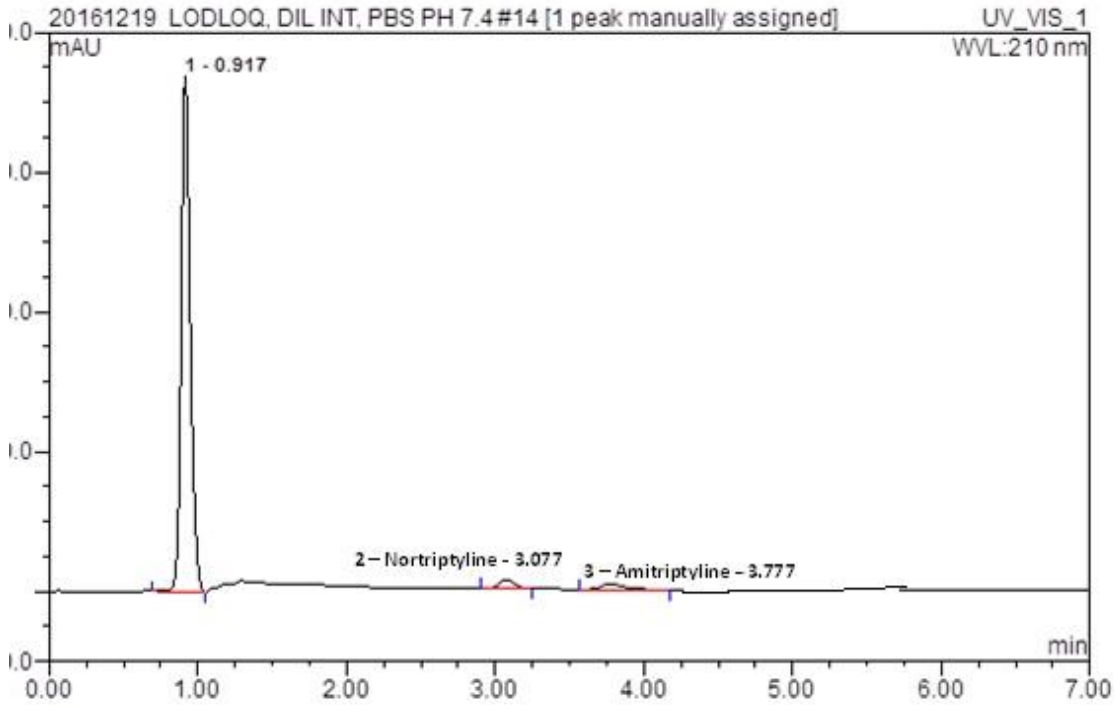

Figure 3.34 Lowest mixed standard (0.078mg/L) of amitriptyline and nortriptyline, from the extended calibration line in PBS pH 7.4

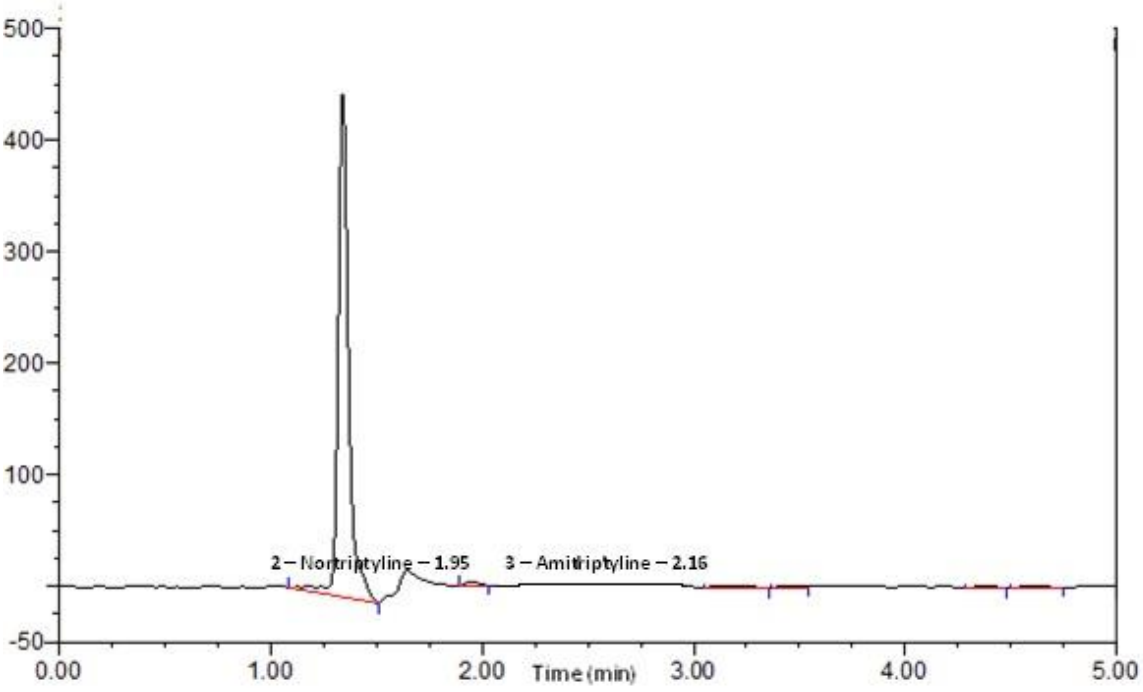

Figure 3.35 Lowest standard (0.078mg/L) of amitriptyline and nortriptyline, from extended calibration line for LOD (pH 5)

3.4.2.3 Precision and Bias

Precision and bias were included in the validation as the precision investigated the closeness of the multiple readings with each other and the bias investigated the similarity of the experimental values with the expected values (SWGTOX, 2013). Precision and bias were measured in these quantitative methods to determine, specifically for precision, the within-day and between-day variation over 5 days with three repeats of the quality control samples per analysis. The calibration standards and QC samples were prepared the same as described previously (See Chapter 2 Section 2.1.5.2) in two different solutions including PBS and AA. The pH 7.4 HPLC method was used for the PBS validation (See Chapter 2 Section 2.2.4.2.1). The pH 5 HPLC method was used for the AA validation (See Chapter 2 Section 2.2.4.2.2). Single factor ANOVA was used to determine the precision and accuracy. The results presented for all three solutions were <20 % and thus within acceptable ranges for further use.

PBS pH 7.4

The buffer present in the solution could result in better ionisation stability of the drugs, as the buffer kept the solution at the correct pH. There would be less chance of the drug changing between ionisation states in the solution. This would allow for most of the drug to be eluted at the same time increasing peak size and reduce peak tailing. ANOVA validation of amitriptyline and nortriptyline in PBS passed, as all precision and bias values were below 20% threshold (See Table 3.8). In addition, the asymmetry and resolution was similar for lowest and highest calibrator showing no loss in peak separation (See Figure 3.36). The values for the asymmetry were 1.50 ± 0.11 and 1.70 ± 0.24 and the resolution values were 3.60 ± 0.21 and 2.92 ± 0.17 for amitriptyline and nortriptyline in water respectively. Amitriptyline and nortriptyline samples in PBS could be run and quantified on HPLC as these samples were validated on the HPLC.

Table 3.8 ANOVA Validation of amitriptyline and nortriptyline in water resulted in results

	amitriptyline	**nortriptyline**
LQC (0.5 mg/L):		
Grand Mean (mg/L)	0.48	0.47
Bias (%)	-4.66	-5.35
Within-Run (%)	1.92	0.79
Between-Run (%)	3.41	2.76
HQC (5 mg/L):		
Grand Mean (mg/L)	4.74	4.66
Bias (%)	-5.26	-6.71
Within-Run (%)	1.41	0.44
Between-Run (%)	3.79	3.66

pH 5

ANOVA validation of amitriptyline and nortriptyline in AA passed, as all precision and bias values were below 20% threshold (See Table 3.9). In addition, the asymmetry and resolution was similar for lowest and highest calibrator showing no loss in peak separation (See Figure 3.37). Amitriptyline and nortriptyline samples in AA could be run and quantified on HPLC as these samples were validated on the HPLC.

Table 3.9 ANOVA Validation of amitriptyline and nortriptyline in water resulted in results

	amitriptyline	**nortriptyline**
LQC (0.5 mg/L):		
Grand Mean (mg/L)	0.50	0.47
Bias (%)	0.28	-6.63
Within-Run (%)	7.30	6.32
Between-Run (%)	12.97	8.03
HQC (5 mg/L):		
Grand Mean (mg/L)	5.11	4.86
Bias (%)	2.21	-2.79
Within-Run (%)	8.64	8.45
Between-Run (%)	11.31	7.56

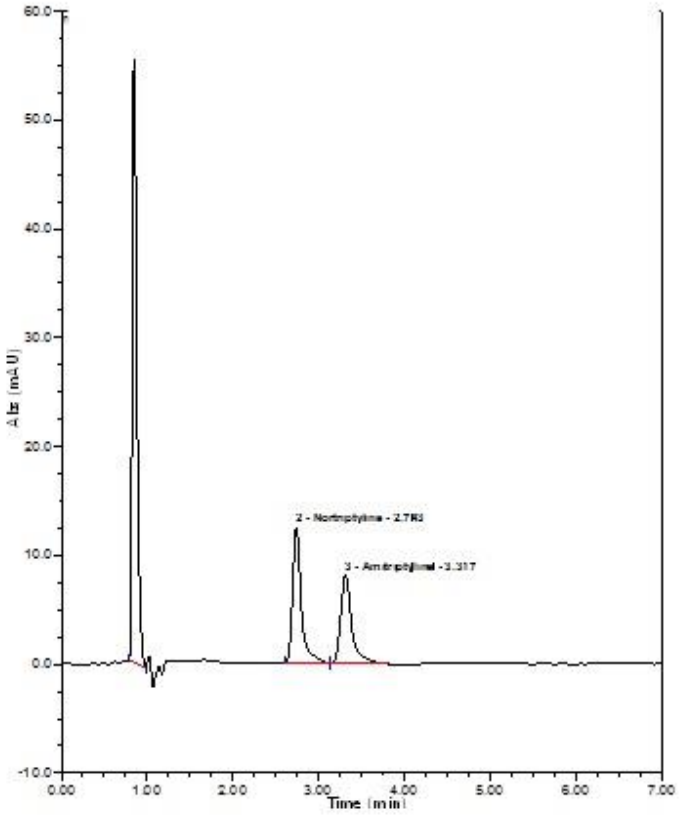

Figure 3.36 Precision and bias, LQC (0.5 mg/L) mixed standard (amitriptyline and nortriptyline) in PBS, analysed on the HPLC

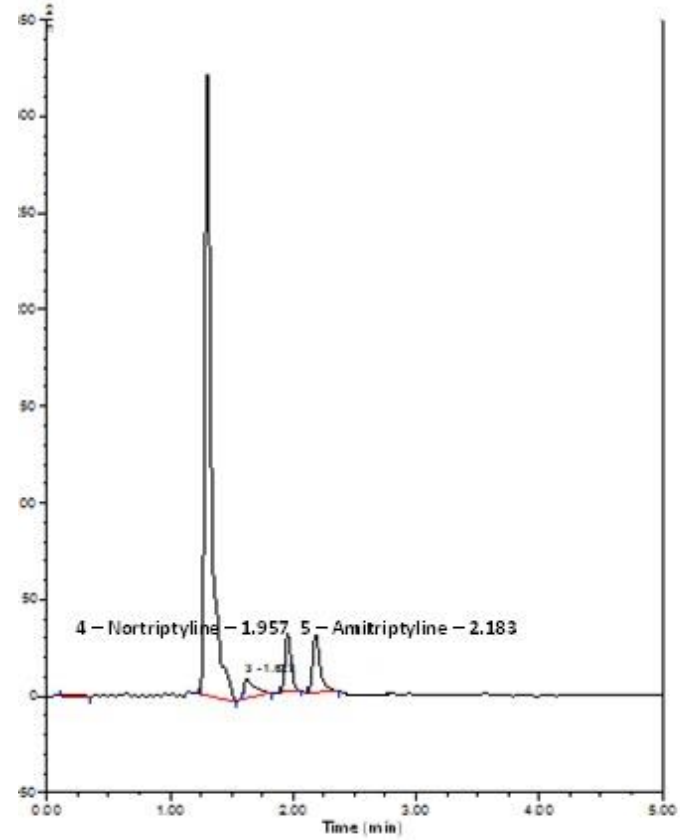

Figure 3.37 Precision and bias, LQC (0.5 mg/L) mixed standard (amitriptyline and nortriptyline) in AA, analysed on the HPLC

3.4.2.4 Stability

This experiment was carried out to determine the stability of the two analytes of interest (amitriptyline and nortriptyline) in both solutions (PBS and AA) at 4°C (fridge temperature) and 37°C (physiological temperature). The length of stability was over 10 days for the analytes in PBS as this would cover the length of the post-mortem period of the individual from the case study (Moriya and Hashimoto, 2001). However, the bladder diffusion studies were carried out over 5 days in accordance with the Skopp paper (Skopp *et al.*, 1997). Therefore, the stability experiment was reduced to a length of 5 days for AA, which was analysed on the HPLC.

3.2.4.2.1 Stability of the antidepressants in PBS pH 7.4

The drug standards for amitriptyline and nortriptyline were prepared in PBS. The humidity chamber was setup at 37°C and 30% humidity; these values were chosen as 37°C was the highest temperature in the research that could have the most effect on the drugs and 30% was the average humidity in the laboratory where the bladder studies were carried out. The drug concentrations of the two QC samples, LQC (0.5 mg/L) and HQC (5 mg/L) were determined using HPLC-DAD methodology. This chamber was equilibrated for half a day. As shown by the graphs (See Figures 3.38 and 3.39), the drugs were within the 20% of the maximum acceptable bias (SWGTOX, 2013) throughout the 10 day experiment. As a result, this shows that the drugs are stable for 10 days.

3.2.4.2.2 Stability of the antidepressants (pH 5)

The drug standards for amitriptyline and nortriptyline were prepared in AA and analysed using HPLC-DAD. This experiment used 3 repeats of each stability sample at both concentrations (LQC – 0.5 mg/L and HQC – 5 mg/L). The solutions were stored in a humidity chamber at 37 ± 0.33 °C the average humidity value in the fume cupboard was 26 ± 5.7 % so this was the value used to set the chamber. The fume cupboard was used to determine humidity as this was where the Franz cell was situated. The peaks were resolved and there was consistency between the three samples for the LQC and HQC stability samples over the 5 days. The amitriptyline and nortriptyline graphs (See Figures 3.40 and 3.41) show the drugs were within the required 20% maximum acceptable bias throughout the experiment and so both drugs, amitriptyline and nortriptyline, are stable over 5 days in ammonium acetate at pH 5.

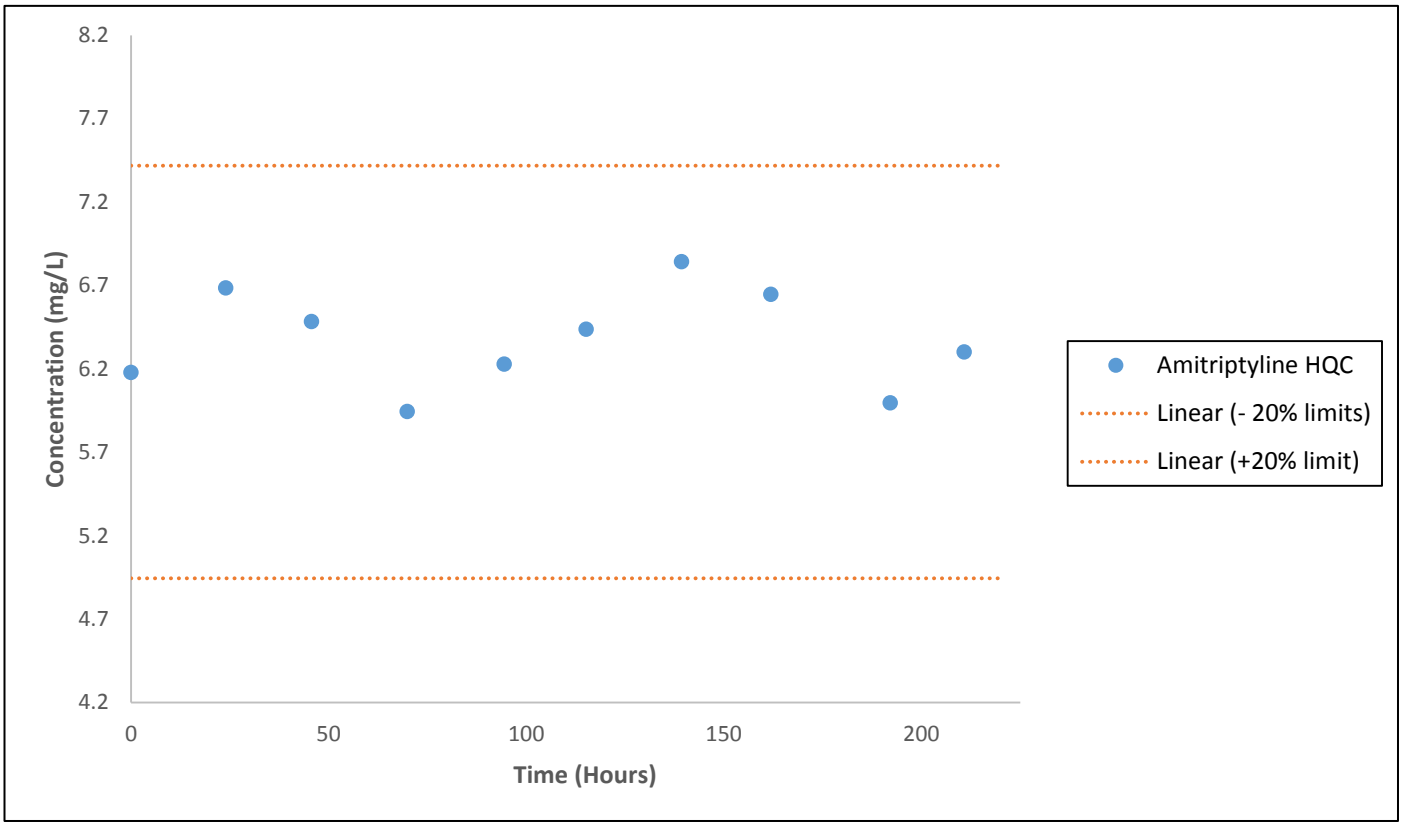

Figure 3.38 amitriptyline HQC (5 mg/L), 10 day stability in PBS at 37°C analysed on the HPLC

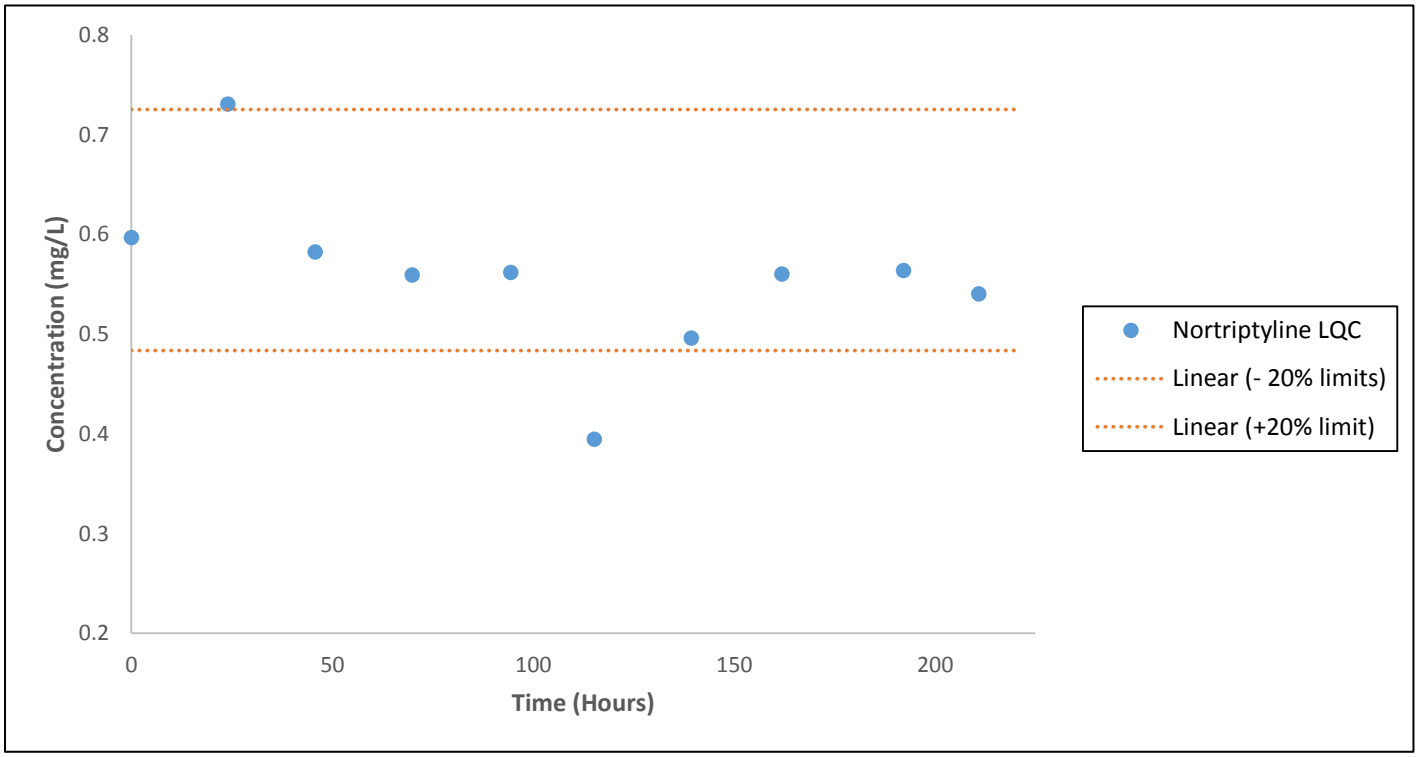

Figure 3.39 nortriptyline LQC (0.5 mg/L), 10 day stability in PBS at 37°C analysed on the HPLC

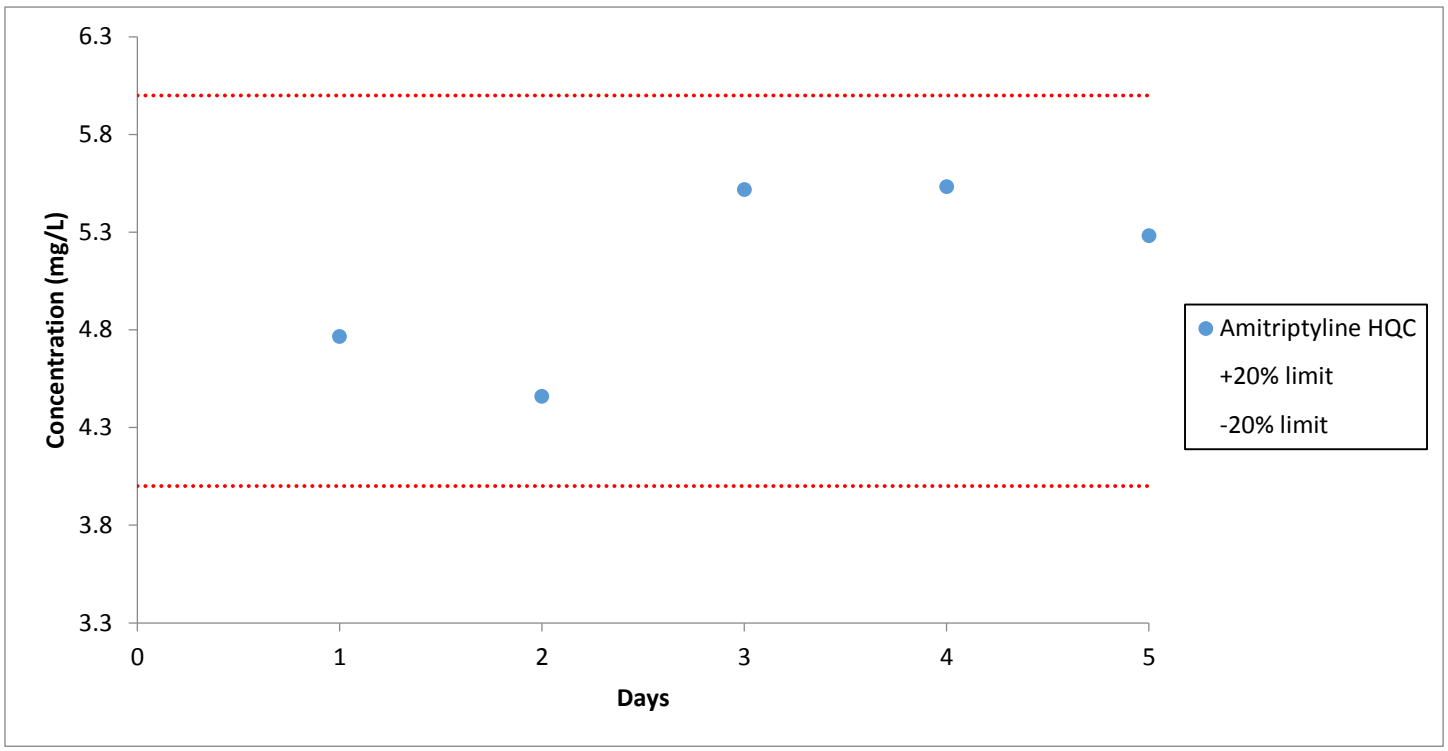

Figure 3.40 amitriptyline HQC (5 mg/l), 5 day stability in AA at 37°C analysed on the HPLC

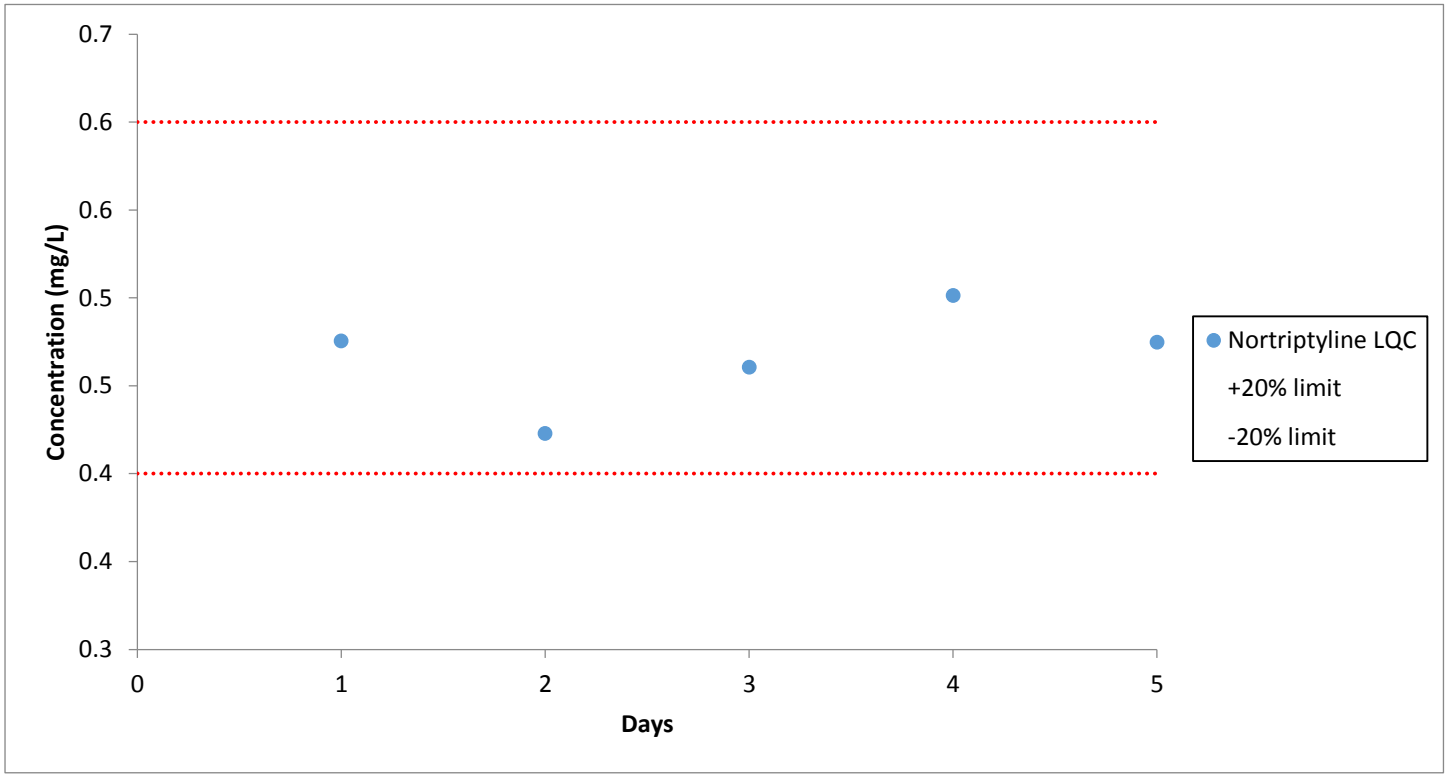

Figure 3.41 nortriptyline LQC (0.5 mg/L), 5 day stability in AA at 37°C analysed on the HPLC

3.4.2.5 Dilution Integrity

During the bladder degradation studies, the absorption of some samples exceeded the top calibration standard, which would prevent from calculating the concentration of the compound with accuracy. Therefore, some samples had to be diluted after initial analysis to reduce the concentration to within the range of the calibration. A number of dilutions were used during the experiments including 1:1, 1:2, 1:10, and 1:20. This experiment was carried out over 5 days with 3 repeats of each concentration to validate the dilutions. The standard calibration and QC samples were prepared using the method from Chapter 2 Section 2.1.5.2. The dilution integrity samples used separate stock solutions of both drugs; the starting stock solutions (10 mg/L and 100 mg/L) were a mixed solution of both drugs. A set of calibration standards (0.16-10 mg/L) and the two QC samples LQC (0.5 mg/L) and HQC (5 mg/L) were prepared and analysed alongside the dilution samples.

The tables for both pH values consist of calculating the concentrations of the diluted samples. The results show that all four dilution factors have consistent results for amitriptyline and nortriptyline. All the values were within the assigned 20% maximum acceptable bias (See Tables 3.10 and 3.11). The average of the three repeats of each dilution factor was calculated then converted from absorbance to concentration using the equation of the line of the calibration standards.

Table 3.10 ANOVA of dilution integrity samples of amitriptyline and nortriptyline in PBS

pH 7.4	1:1 (5mg/L)	1:2 (3.33mg/L)	1:10 (1mg/L)	1:20 (5mg/L)
amitriptyline:				
Grand Mean (mg/L)	8.40	8.56	10.03	100.08
Bias (%)	-16.04	-14.37	0.27	0.08
With-in Run (%)	5.83	6.67	4.46	11.51
Between-Run (%)	14.60	9.42	10.71	18.45
nortriptyline:				
Grand Mean (mg/L)	10.70	10.11	11.31	108.04
Bias (%)	7.01	1.09	13.12	8.04
With-in Run (%)	4.28	6.80	3.88	5.29
Between-Run (%)	12.59	15.90	14.27	17.18

Table 3.11 ANOVA of dilution integrity samples of amitriptyline and nortriptyline in AA

pH 5	1:1 (5mg/L)	1:2 (3.33mg/L)	1:10 (1mg/L)	1:20 (5mg/L)
amitriptyline:				
Grand Mean (mg/L)	8.45	8.43	9.50	99.32
Bias (%)	-15.51	-15.72	-4.97	-0.68
With-in Run (%)	11.52	12.62	1.02	0.27
Between-Run (%)	16.89	14.39	1.50	**0.75**
nortriptyline:				
Grand Mean (mg/L)	9.74	9.20	10.02	112.50
Bias (%)	-2.64	-7.97	0.22	12.50
With-in Run (%)	7.94	13.96	8.73	5.69
Between-Run (%)	15.21	14.93	13.63	15.86

3.4.2.7 Interference/Carryover

This study involved obtaining a number of blank solution chromatograms that were analysed directly after the highest calibration standard (10 mg/L) on the HPLC from previously completed experiments. These were taken to determine if there was any carryover of drugs from this highest standard to the blank solution. If there was no carryover then this would suggest that the method was suitable for the drugs as there was no drug retention past the expected sample run time also there was no evidence of any column degradation. In addition, a stability experiment was carried out on blank PBS and AA to determine if the solutions are stable over 10 days.

PBS pH 7.4

The blank stability solutions prepared involved 20 mM PBS and AA that were analysed in triplicate and stored in the humidity chamber at 37°C for 5 days to mimic the bladder studies. Samples removed once daily to sample for analysis on the HPLC. The samples were shaken before the 1 ml sample was removed by a 1 ml syringe and transferred to an 8 ml glass vial. The HPLC method used for the analysis was pH 7.4 (See Chapter 2 Section 2.2.4.2.1). The chromatograms showed no carryover of any drugs from the highest standard (See Figure 3.44) to the blank solution (See Figure 3.45). The HPLC results of the stability blank solutions show there was an interference peak at 2.3 minutes in the blank PBS solution (See Figure 3.46) with a grand average absorbance value of 1.24 ± 0.31 mAU. However, this does not reach 2 mAU units, so this is a small interference peak.

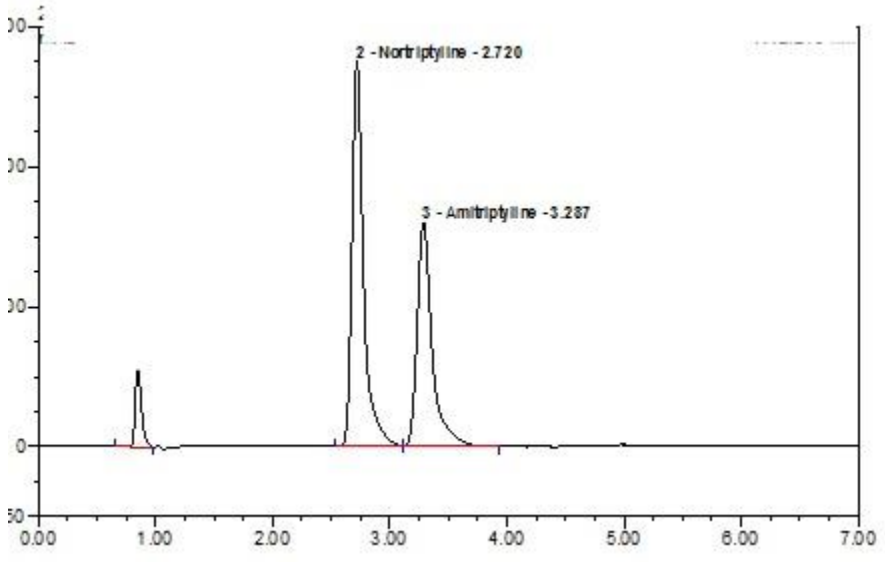

Figure 3.42 10mg/L standard from pH 7.4 validation

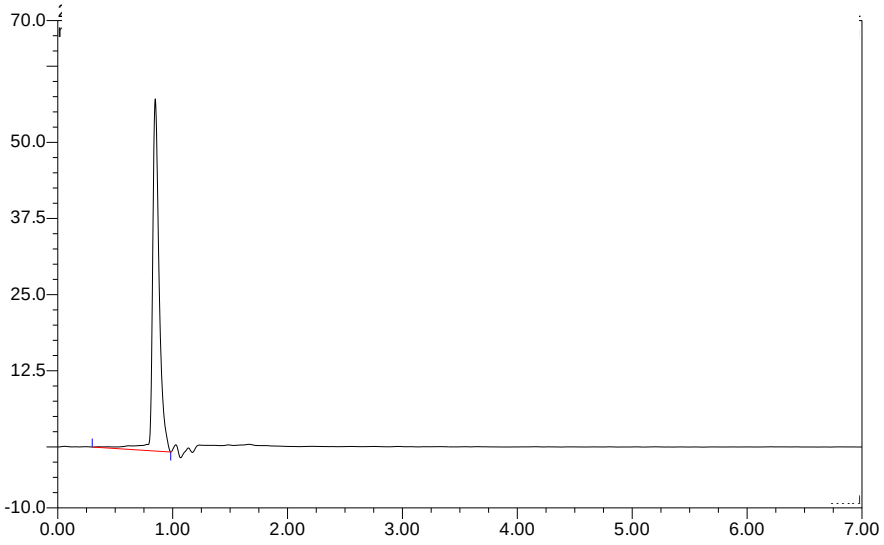

Figure 3.43 Blank PBS sample from pH 7.4 validation

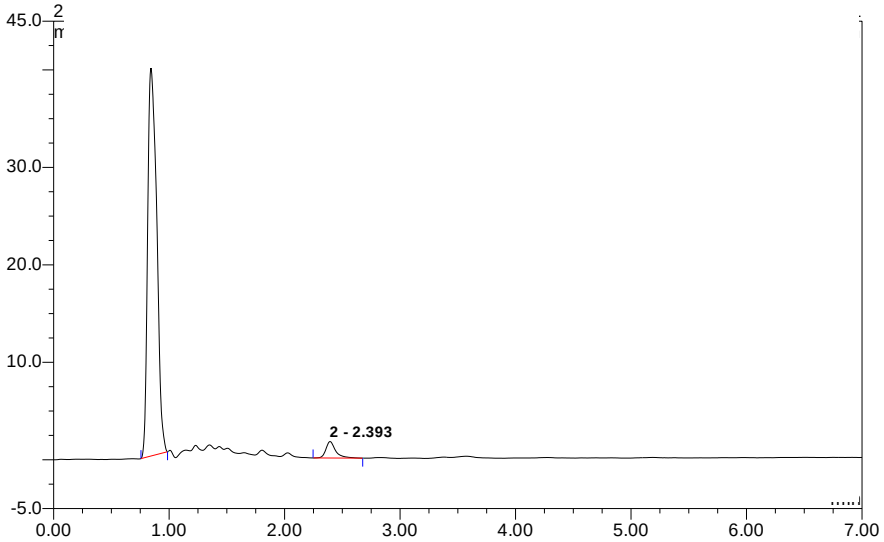

Figure 3.44 Largest peak height of interference peak images

AA pH 5

In order to determine the stability of blank pH 5 solution on the HPLC over 5 days. The pH 5 HPLC method was used to analyse these samples (See Chapter 2 Section 2.2.4.2.2). The humidity chamber for the 37°C experiments had an average humidity value in the fume cupboard of 26%, determined using a hygrometer (Fisher Scientific, Traceable Humidity/Temperature Pen with Memory). The temperature was consistent throughout the whole experiment. The humidity chamber was left to equilibrate for half a day. Three large glass vials were filled with 10 ml of blank AA each, sealed with Parafilm and placed in the humidity chamber. The chromatograms showed no carryover of any drugs from the highest standard (See Figure 3.47) to the blank solution (See Figure 3.48). The blank AA stability solution results show a small peak over the 5 days with an average peak height of 2.851 ± 0.3 mAU at 2.2 minutes (See Figure 3.49). This is a similar retention time to amitriptyline at 2.1 minutes. This could be a possible interfering peak in the blank solution. However, it does not seem to increase over the 5 days so it is not a cumulative interfering peak.

Both pH solutions

The ten-day interference study showed small interference peaks for both pH 5, the PBS interference peaks do not pass 2 mAU and the AA interference peak height was 2.8 mAU. The interference peaks had a similar retention time to amitriptyline of approximately 2.25 minutes. This retention time would co-elute with amitriptyline, this could affect the quantification of the drug. However, the peak height was not significant to affect the quantification as amitriptyline was validated in both pH solutions.

3.5 Conclusion

The method development of the Franz cell with the bladder tissue, *in vivo* rat study, and the instrumental methods (UV and HPLC) involving the drugs used in the bladder studies was a success. The prevention of leakage from the Franz cells, catheterising the rat with a CT visible drug and the ability to secure the rat for scanning, and also identifying and quantifying the drugs on the instruments. In addition, the validation of the model compound (rhodamine B) and the two antidepressant drugs (amitriptyline and nortriptyline) were successful in the respective solutions (pH 7.4 and pH 5) that were used in the bladder studies. A number of parameters were included in the validations to include all eventualities in the method including linearity, LOD/LOQ, precision and bias, sample stability, dilution integrity and interference/carryover. The success of these method developments and validations allows the studies to be carried out.

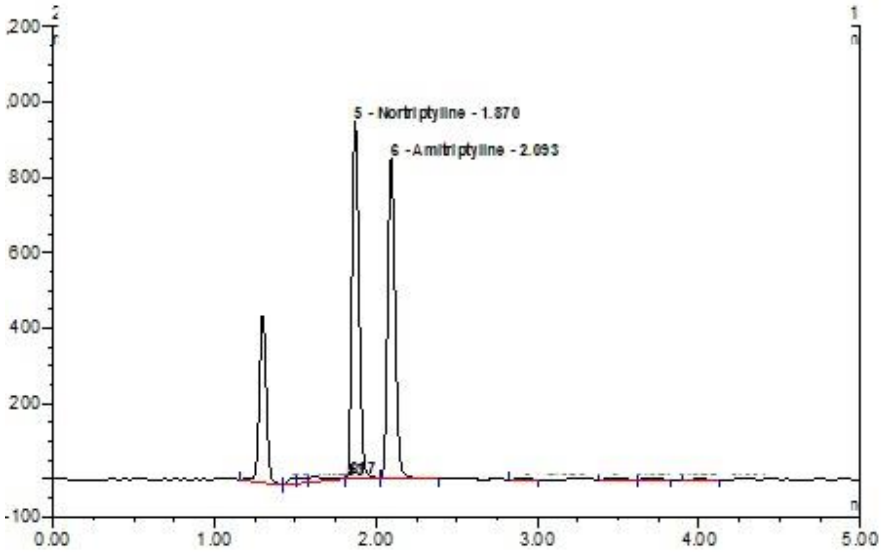

Figure 3.45 10 mg/L mixed standard (amitriptyline and nortriptyline), pH 5 validation

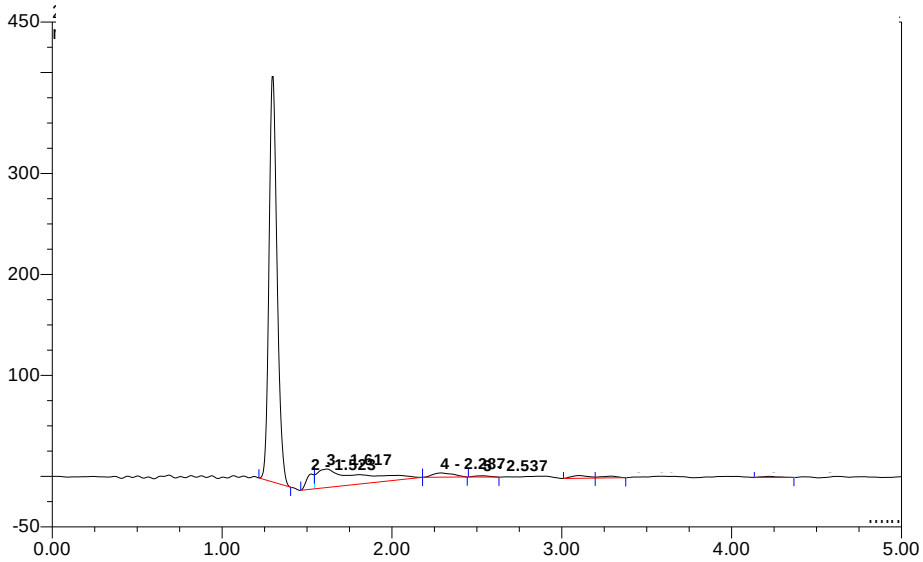

Figure 3.46 Blank AA sample from pH 5 validation

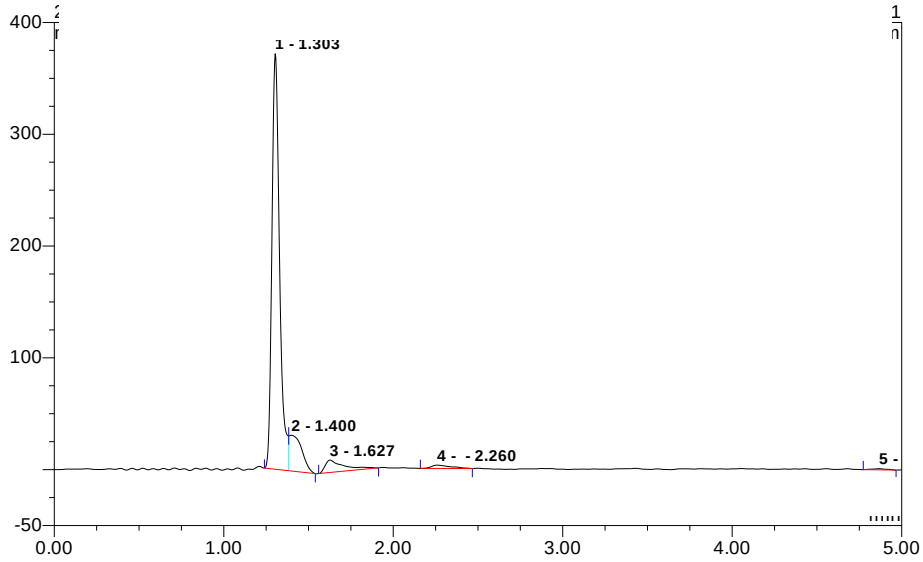

Figure 3.47 Interfering peaks in the interference study

Chapter 4 – Diffusion of Drugs from the Bladder using Franz cells

4.1 Introduction

Post-mortem redistribution (PMR) has been shown to occur, via the comparison of sampling sites overtime, in a number of organs including heart, lungs, liver and stomach to different degrees due to accumulation of the drugs in the organs (Hilberg *et al.*, 1992; Hilberg, Mørland and Bjørneboe, 1994; Pounder, Anderson and Watmough, 1994; Brunet, Hauet and Hébrard, 2010). Each organ has a specific biological task within the body that requires different organ structures that are optimal for that activity to sustain life. In relation to the bladder, the structure is multiple layers of cells that are ultimately layered with the mainly impenetrable urothelium (Grasso and Calderón, 2009). This allows the urine to be collected and stored in the bladder, isolated from the rest of the body until it can be eliminated. However, after death the functions of the organs cease, starving the cells of oxygen and nutrients ultimately irreversibly damaging the tissues. This damage can be observed in different ways including the breakdown of the tissues. The breakdown results in the release of urine from the bladder and tissue bound compounds into the body cavity. The drugs whether free or bound to other compounds are then free to diffuse through surrounding fluids and tissues. However, the ionisation state of a drug would affect the ability of the drug to diffuse through tissues, which would result in this becoming a factor in the extent of PMR from the bladder. Therefore the pH of the post-mortem urine, which has a range of between 5.5 – 8.0 (Jones and Karlsson, 2005), would have to be taken into consideration as this would affect the ionisation state of the drug. Bladder tissue consists of a specific set of cells that are different from other organs and so would need to look into PMR at this site to determine how quickly the tissues breakdown after death. It has been suggested there may be diffusion from the bladder when there are high drug concentrations contained in the bladder (Moriya and Hashimoto, 2001).

For the initial investigation, the number of variables would need to be limited to have a controlled environment including temperature, solution pH and tissue degradation. After death, many changes occur including cooling of the body and anaerobic respiration. The latter contributes to the pH reduction creating a more acidic environment directly resulting in the degradation of tissues. Using a range of temperatures and solution pH a comparison could be made between physiological and post-mortem conditions for a number of drugs in relation to the concentration of the drugs that could diffuse through bladder tissue during a set period. This would involve using sections of tissues to allow for an acceptable number of repeats that would be used to create a reproducible method. However, a parameter that cannot be controlled is the bladder thickness as each bladder would be in different states after death including volume of urine present. This would affect the thickness of the bladder membrane and the thickness could be a factor that affects the amount of drug that could diffuse through the bladder tissue, as the thicker the tissue membrane the longer it could take the drug to diffuse. A model compound can be used to visualise the diffusion of the drug through the tissue. A study used a xanthene dye (rhodamine B) as a model, then analysed the diffusion of morphine in a method that mimics passive diffusion through the wall of a vein. The methodology involved using Franz cells to allow contact of the dye with the tissue. The solution was sampled over a period and the absorbance of the dye in the acceptor chamber was measured with a UV-visible spectrophotometer (Skopp *et al.*, 1997).

Different temperatures were shown to affect the diffusion with the lower temperature (4°C) having an increased lag time at the start of the experiment than at room temperature (20°C) (Skopp *et al.*, 1997). This shows the lower temperature reduces the concentration of the drug over the same period, therefore less drug

diffusing through the tissue results in less drug to redistribute after death. Using this study as a guide rhodamine B could be used as the model compound in this research.

There is a possibility of the drugs having an affinity to the biological tissue; this could be a factor that reduces the diffusion of a drug from the bladder. The affinity of a drug for biological tissue determines if the drug is preferentially absorbed into the tissue rather than stay in solution. Calf arteries were used to determine equilibration of a hydrophobic compound, paclitaxel, within 72 hrs and also the affinity of the drug for the tissue. The results found that the drug had high affinity for the artery, as the concentration was higher in the tissue than in the surrounding solution. Different concentrations were shown to have no effect on the partitioning of the drug into the tissue, which was not uniform showing preferential binding at different layers within the artery. Endovascular and perivascular introduction of the drug into the artery was undertaken and the higher concentration was found in the corresponding section. High affinity for the artery was determined as there was accumulation in the tissue as the drug was passed through the intact lumen of the artery (Creel, Lovich and Edelman, 2000). This is relevant to this study as the drug could preferentially accumulate in the bladder tissue rather than diffuse through into the acceptor chamber, which would result in lower drug concentrations.

There are a limited amount of studies carried out on the degradation of the bladder and the affect this has on the permeability of the tissue to drugs. During life, cells are replaced and therefore the membranes are replaced without any loss in function. However, when there is damage this has been shown to increase permeability of bladder tissue. Compounds can permeate further into the bladder tissue due to the removal of the umbrella cells making up the urothelium. This shows that the urothelium is the main barrier between the bladder contents and the rest of the body. Damage resulting from disease can disrupt the bladder membrane in the case of interstitial cystitis. This inflames the bladder membrane and can disrupt the impenetrable barrier. An animal study mimicked the symptoms of cystitis in guinea pig bladders and the results showed an increase in the water and urea permeability through the bladder membrane up to 24 hrs post disruption (Lavelle *et al.*, 1998). Another study used protamine sulfate, which has been shown to disrupt only the urothelium in the bladder membrane and this has shown to increase the permeability of the bladder for urea. The bladder contents can access the lower layers of the membrane as the disruption can extend to the lamina propria. After 40-60 minutes of ischemia both dyes used to show the extent of permeation could be detected in the bladder membrane and this concentration increased with increasing time of ischemia (Koroäec and Jezernik, 2000). This determines what occurs within the initial stage after death, that there is damage to the umbrella cells within the lumen of the bladder. These cells disconnect from the rest of the membrane allowing for the urine that could contain large concentrations of drugs diffuse through into the lower layers of the bladder membrane. Combining the *in vitro* studies with bladder tissue there have been studies investigating the passive diffusion of a number of drugs through intact bladder membranes (Borzelleca, 1965) including barbiturates, basic drugs (atropine, neostigmine and physotigmine) (Borzelleca, 1959) and nicotine (Borzelleca, 1963).

The antidepressant amitriptyline is a first generation tricyclic antidepressant that was first introduced in 1961 (Baselt, 2008). This drug is still found in many cases in both the therapeutic and toxic range and as

amitriptyline is mainly metabolised to nortriptyline we included this in the study. As it is a well-studied drug in the post-mortem environment (Hilberg *et al.*, 1992, 1993; Hilberg, Mørland and Bjørneboe, 1994), it would be suitable to use in the investigation of diffusion from the bladder.

4.2 Aims

The aims of this chapter was to determine how quickly the bladder broke down after death and also the speed of diffusion of three model compounds (rhodamine B, amitriptyline and nortriptyline) using an *in vitro* model over the course of 5 days. The effect of a number of relevant parameters was studied including bladder thickness, solution pH, drug concentration tissue degradation and temperature.

4.3 Results

Porcine bladder tissue sections were used in conjunction with Franz cells to determine if a number of parameters (drug concentration, solution pH, temperature, tissue degradation, and bladder thickness) had a significant effect on drug diffusion using three drugs, two in conjunction together, including rhodamine B and two antidepressant drugs amitriptyline and nortriptyline. Franz cells were used to study the diffusion of either 100 mg/L rhodamine B or a mixed standard of 100 mg/L amitriptyline and nortriptyline. The rhodamine B was used as a model compound and the drug mixture were the analytes of interest in this research. The two solution pH values were pH 7.4 (representing physiological pH) and pH 5 (post-mortem pH (Donaldson and Lamont, 2013)), which are within the wide range of pH values that have been found in PM urine (pH 4.6 – 8.5) (Cook, Strauss and Caplan, 2007). Three temperatures were included for comparison including 37°C, physiological body temperature, 20°C, room temperature to which the body equilibrates after death and 5°C the mortuary fridge temperature. Tissue degradation was compared using fresh bladders and degraded bladders, which were thawed after being frozen for one week prior to the analysis. In order to see if there may be different rates of diffusion from the same bladder 4 sections were taken from the same bladder, resulting in a set of intra-bladder results. The control experiments, bladder sections with donor chamber of blank PBS and AA solutions, had no significant effect on the results with average results below the method limit of detection (data not shown).

4.3.1 Influence of temperature on the amount of drugs diffusing through bladder sections

After death, the body cools down therefore over time the temperature within the body changes (Leinbach, 2011). Bladder sections were equilibrated to three different temperatures to determine if temperature has a significant effect on the rate of drug diffusion, compared between two different pH values (pH 7.4 and 5) and tissue degradation levels (fresh and degraded). The temperatures studied were: 37°C, 20°C, and 5°C, which represent physiological body temperature, room temperature and mortuary fridge temperature respectively.

4.3.1.1 Rhodamine B

Three temperatures were used to equilibrate the bladder tissue to determine the effect of the diffusion of 100 mg/L rhodamine B solution in pH 7.4 and pH 5. Three types of bladder tissue were used that included fresh bladder sections from different bladders (inter-bladder), four sections from the same bladder (intra-bladder) and degraded bladder sections (inter-bladder). The highest cumulative concentration of rhodamine B was 3.46 ± 2.72 mg/L at 37°C from the intra-bladders in pH 5 (See Table 4.1). The lowest cumulative rhodamine B concentration was at both pH values at 5°C from the fresh inter-bladder experiments and 20°C (pH 5) using fresh intra-bladders with results below the limit of detection of the rhodamine B method (See Figure 4.1),

4.3.1.1.1 Temperature comparison of rhodamine B for the bladder sections

The differences between the cumulative concentrations of rhodamine B was determined in the later stages of the experiments, with main differences between 37°C and 20°C and also 37°C and 5°C (See Figures 4.1 – 4.6). The fresh inter-bladders in pH 7.4 buffer solution showed a significant difference between both the sets of temperatures at 94 ± 0.1 hrs with more diffusion from 37°C in both cases ($p < 0.001$); however, no difference was observed between 20°C and 5°C (See Figure 4.7). The peak cumulative concentrations were 1.70 ± 1.0 mg/L at 37°C, 0.67 ± 0.71 mg/L at 20°C and a concentration below the limit of detection for 5°C. The significant difference in the cumulative rhodamine B concentrations was present in the fresh inter-bladders at pH 5 buffer solution at 100 ± 0.06 hrs between 37°C and 20°C and also 37°C and 5°C. The peak cumulative concentrations were 2.29 ± 1.22 mg/L at 37°C, 0.85 ± 0.89 mg/L at 20°C and a concentration below the limit of detection for 5°C. As expected from the Fick equation there was a greater amount of diffusion present at the highest temperature in both instances ($p > 0.0001$); similar results were found between 20°C and 5°C (See Figure 4.8). The peak cumulative rhodamine B concentrations for the fresh inter-bladders at the three temperatures are shown in table 4.1. The degraded inter-bladders also showed significant difference in both pH solutions at 100 ± 0.08 hrs and 99 ± 0.06 hrs for pH 7.4 (p values less > 0.001) and pH 5 ($p > 0.05$) respectively. There were similar results between the two pH solutions at the highest temperature (37°C), which was significantly different from both 20°C and 5°C. However, there was no difference between the two lower temperatures with similar cumulative concentrations at 20°C and 5°C (See Figures 4.9 and 4.10). The peak cumulative concentrations at pH 7.4 were 2.63 ± 2.46 mg/L at 37°C, 0.44 ± 0.33 mg/L at 20°C and 0.54 ± 0.46 mg/L for 5°C. The peak cumulative concentrations at pH 5 were 1.45 ± 1.26 mg/L at 37°C, 0.58 ± 0.42 mg/L at 20°C and 0.31 ± 0.49 mg/L for 5°C. The intra-bladders showed slightly different results with a significant difference only between 20°C and 5°C in pH 7.4 at 96 ± 0.03 hrs ($p > 0.01$) (See Figure 4.11). The peak cumulative concentrations were 1.86 ± 1.75 mg/L for 37°C, 2.73 ± 2.31 mg/L for 20°C and a concentration below the limit of detection for 5°C. The lower pH (pH 5) showed similar significant difference as the inter-bladder experiments with differences between 37°C and the two lower temperatures at 100 ± 0.04 hrs ($p < 0.0001$) (See Figure 4.12). The peak cumulative rhodamine B concentrations were 3.46 ± 2.72 mg/L at 37°C and concentrations below the limit of detection for both 20°C and 5°C. Overall, this shows temperature would have a significant effect on the cumulative concentration of rhodamine B, with the range of differences between 37°C and 20°C between 0.87 – 3.46 mg/L, diffusing through bladder tissue over approximately 100 hrs (~ 4 days) after death.

4.3.1.1.2 pH comparison of rhodamine B for the bladder sections

There was slightly more diffusion, with an increase of 0.59 mg/L rhodamine B, for the fresh inter-bladder at pH 5. However, the standard deviation for all the cumulative rhodamine B concentrations was large and therefore the concentrations would overlap. This is demonstrated with the cumulative concentrations graphs, which show similar diffusion patterns at both pH (See Figures 4.1 and 4.2). Similar rhodamine B cumulative concentrations were shown for the degraded bladders, with no increase in cumulative concentration at the two lower temperatures. However, at 37°C there were higher cumulative concentrations occurring at the later stages of the experiment at pH 7.4 (See Figures 4.3 and 4.4). The intra-bladders had different amounts of diffusion dependant on both temperature and pH as 20°C showed more diffusion than 37°C in PBS pH 7.4, whereas at pH 5 only 37°C showed any rhodamine B diffusion (See Figure 4.5 and 4.6). Overall, it shows that there is an increase in cumulative concentration of rhodamine B at the highest temperature and the higher pH, showing there is a possibility of pH affecting the amount of drug diffusing through the tissue. These results oppose the expected percentage of ionisation of rhodamine B, which should limit the diffusion with 99.9% at pH 7.4 and 86% ionised at pH 5 of the carboxylic acid group. However, the quaternary group would still be ionised. Less diffusion would be expected at the higher pH, which shows that this is not the main factor that would limit rhodamine B diffusion.

4.3.1.1.3 Other comparisons of rhodamine B for the bladder sections

The tissue degradation does not have a significant effect on the diffusion of rhodamine B with similar results to the fresh bladders. The cumulative concentration graphs are similar to the respective pH solutions using fresh bladders (See Figures 4.1 – 4.6). One aspect that is dissimilar is the peak cumulative rhodamine B concentrations at 5°C, both sets of fresh bladders have concentrations < the limit of detection of the rhodamine B method. However, the degraded bladders show an increased amount of diffusion to the point where the concentrations are within the lower section of the calibration range (See Table 4.1).

The significant differences of the cumulative concentrations of rhodamine B between the temperatures only showed at the later stages of the experiment with the shortest post-mortem interval of 94 ± 0.1 hrs. This shows the highest temperature (37°C) only show increased concentrations in comparison with the lowest temperatures (20°C and 5°C) after at least 4 days post-mortem (See Figure 4.7).

Table 4.1 Peak cumulative concentrations of rhodamine B in both pH solutions from the bladder studies within 100 hrs after death

	Peak cumulative rhodamine B concentration (mg/L)	
Experiment	**pH 7.4**	**pH 5**
Fresh Inter-bladder		
37°C	1.70 ± 1.0	2.29 ± 1.22
20°C	0.67 ± 0.71	0.85 ± 0.89
5°C	< LOD	< LOD
Degraded Inter-bladder		
37°C	2.63 ± 2.46	1.45 ± 1.26
20°C	0.44 ± 0.33	0.58 ± 0.42
5°C	0.54 ± 0.46	0.31 ± 0.49
Fresh Intra-bladder		
37°C	1.86 ± 1.75	3.46 ± 2.72

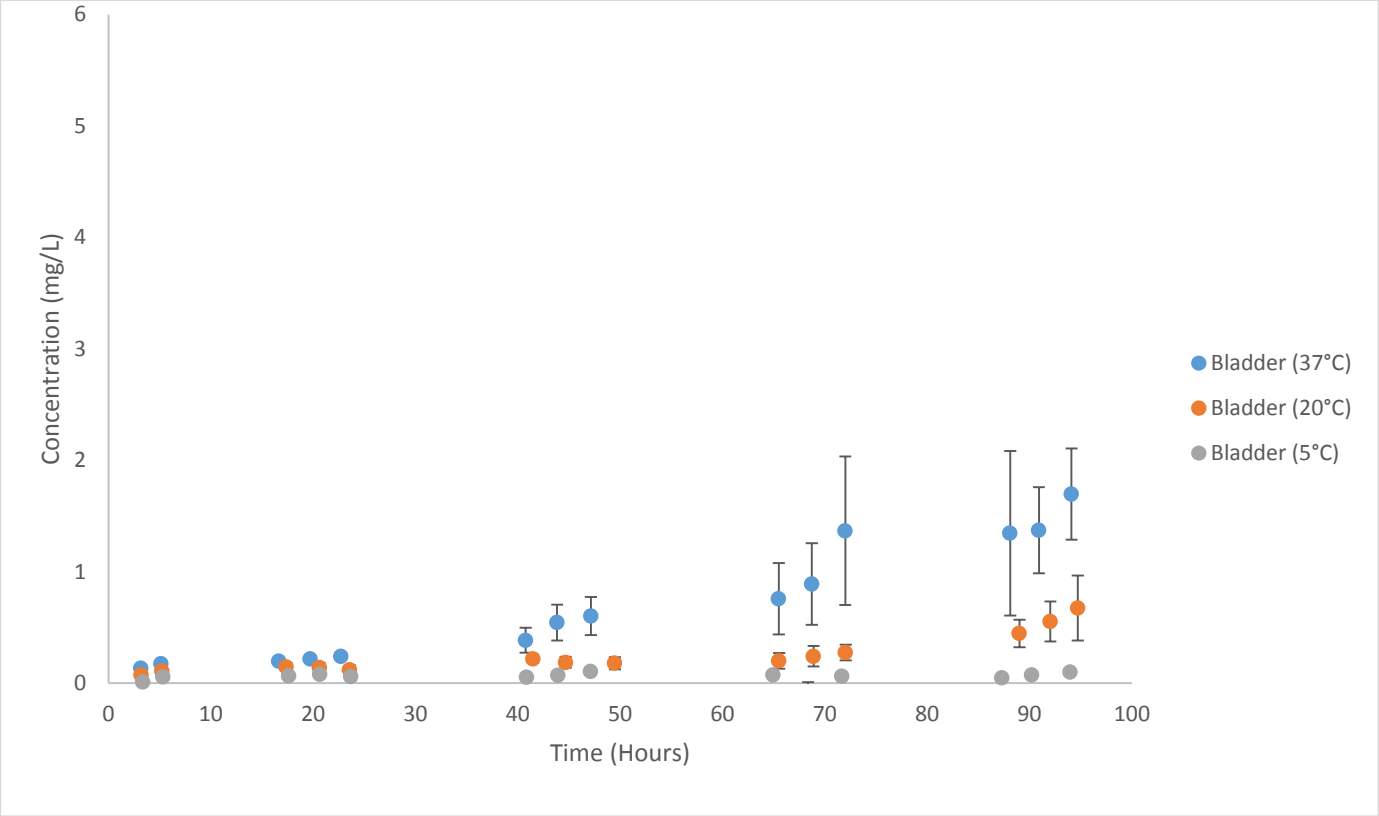

Figure 4.1 Average rhodamine B cumulative concentration with six fresh inter-bladder sections, comparing bladder temperatures at 37, 20 and 5°C in pH 7.4 (n=6) (Error bars are ± S.D.)

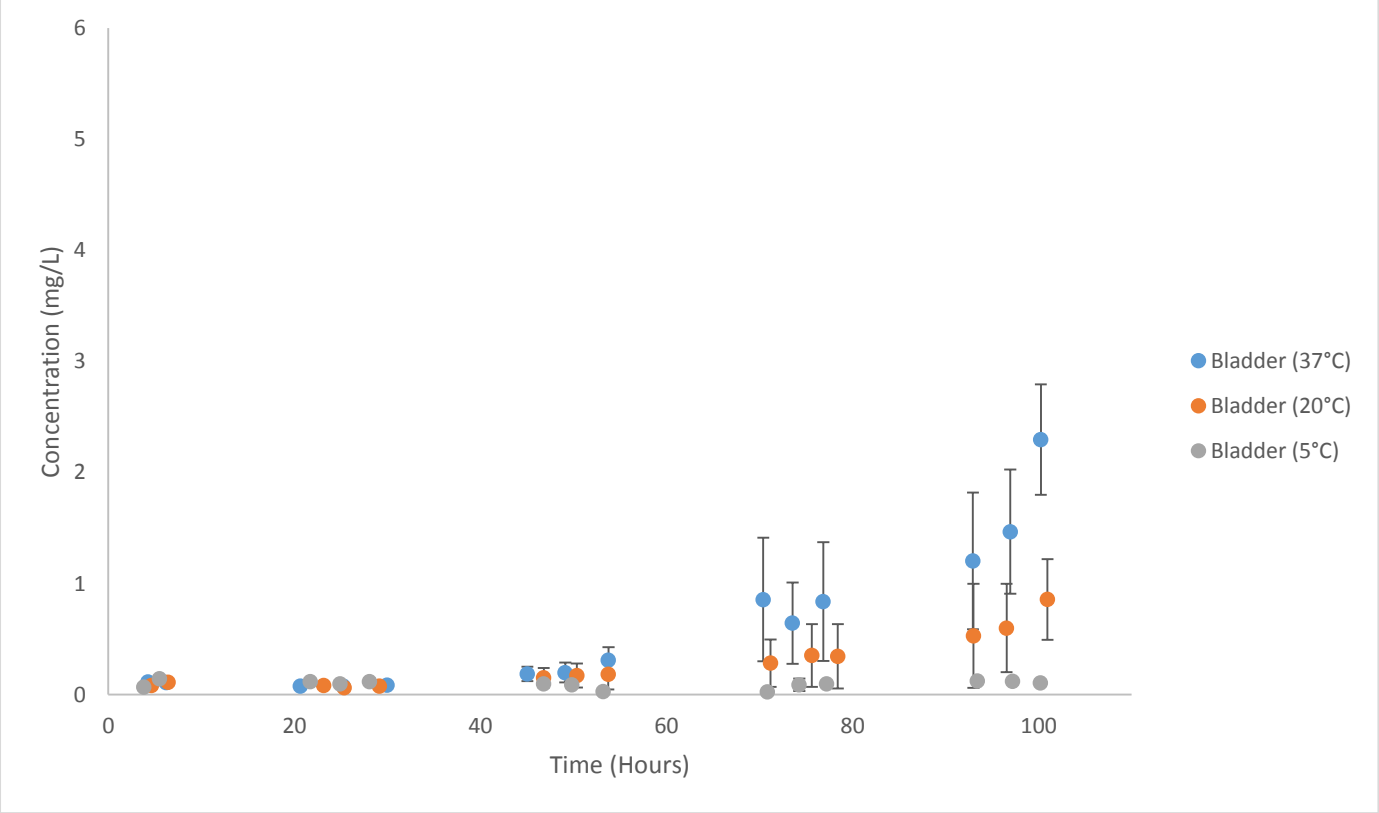

Figure 4.2 Average rhodamine B cumulative concentration with six fresh inter-bladder sections, comparing bladder temperatures at 37, 20 and 5°C (pH 5) (n=6) (Error bars are ± S.D.)

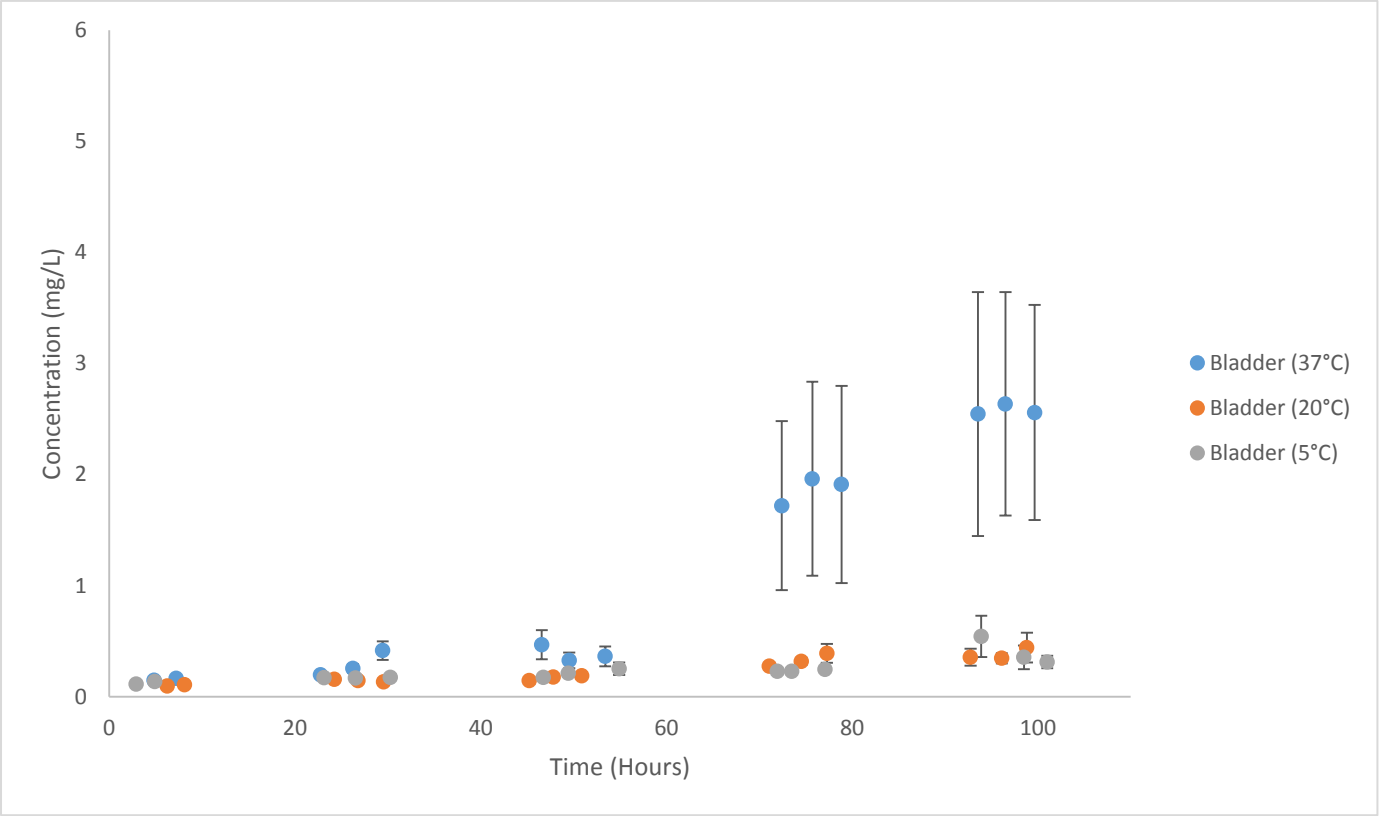

Figure 4.3 Average rhodamine B cumulative concentration with six degraded inter-bladder sections, comparing bladder temperatures at 37, 20 and 5°C in pH 7.4 (n=6) (Error bars are ± S.D.)

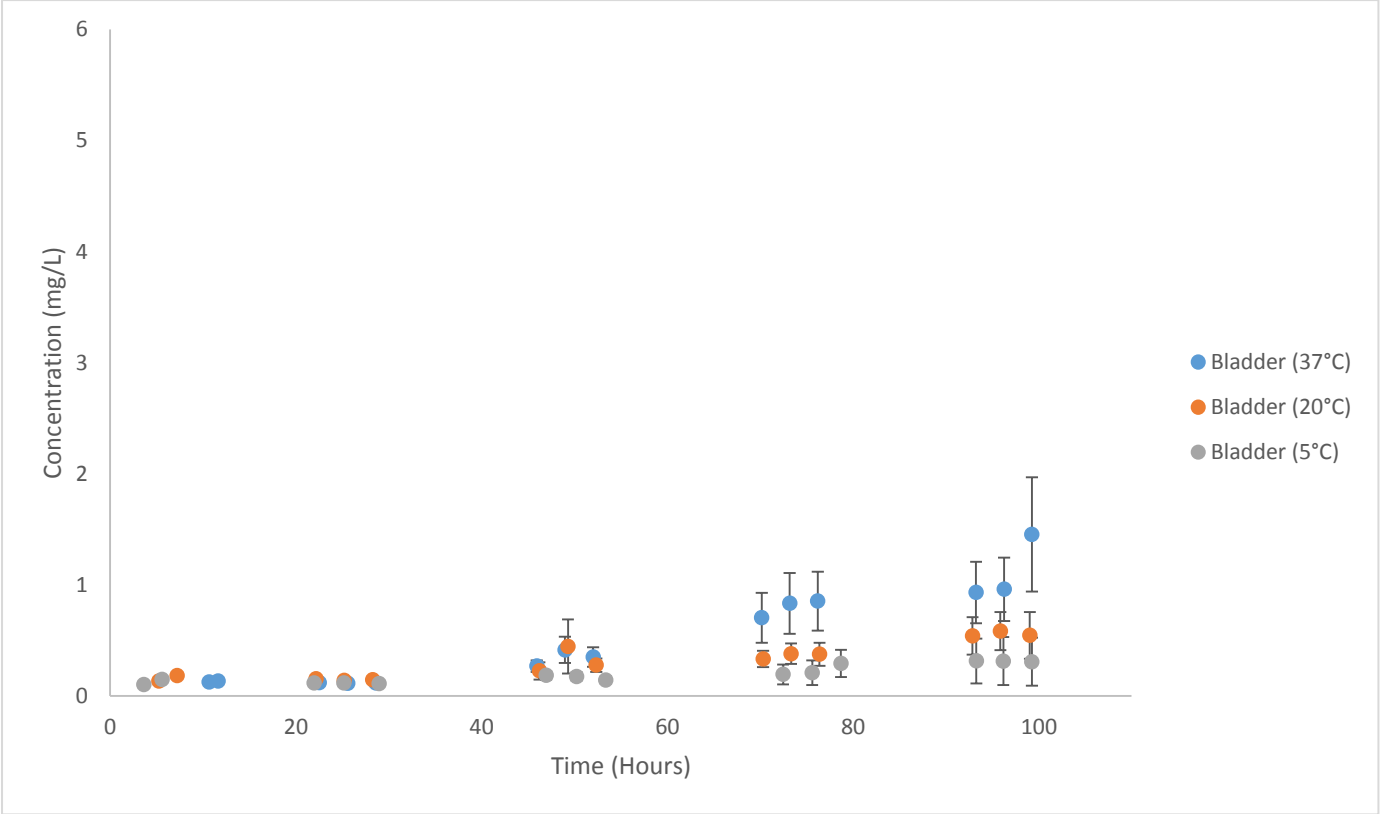

Figure 4.4 Average rhodamine B cumulative concentration with six degraded inter-bladder sections, comparing bladder temperatures at 37, 20 and 5°C (pH 5) (n=6) (Error bars are ± S.D.)

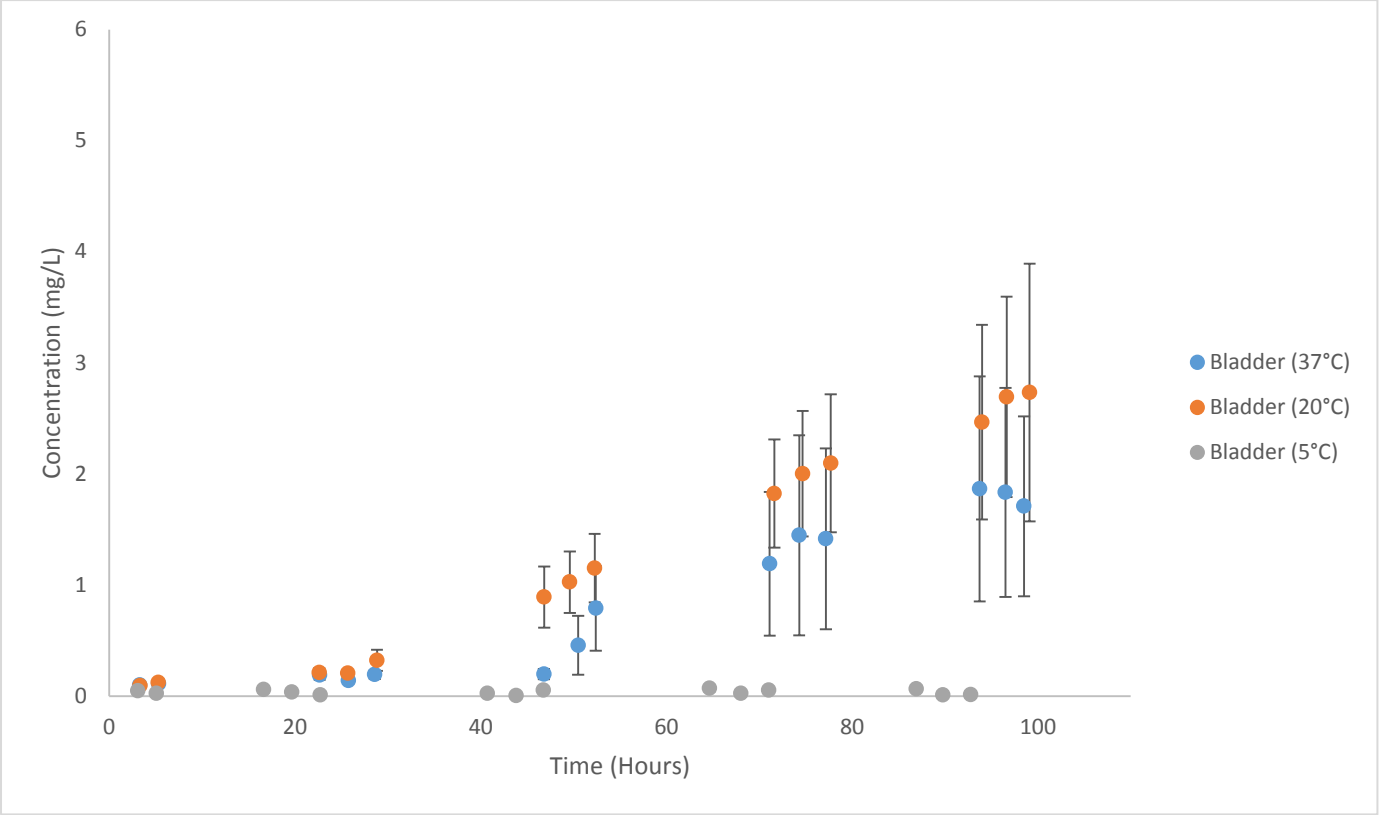

Figure 4.5 Average rhodamine B cumulative concentration with four sections from one bladder (intra-bladder sections), comparing bladder temperatures at 37, 20 and 5°C in pH 7.4 (n=4) (Error bars are ± S.D.)

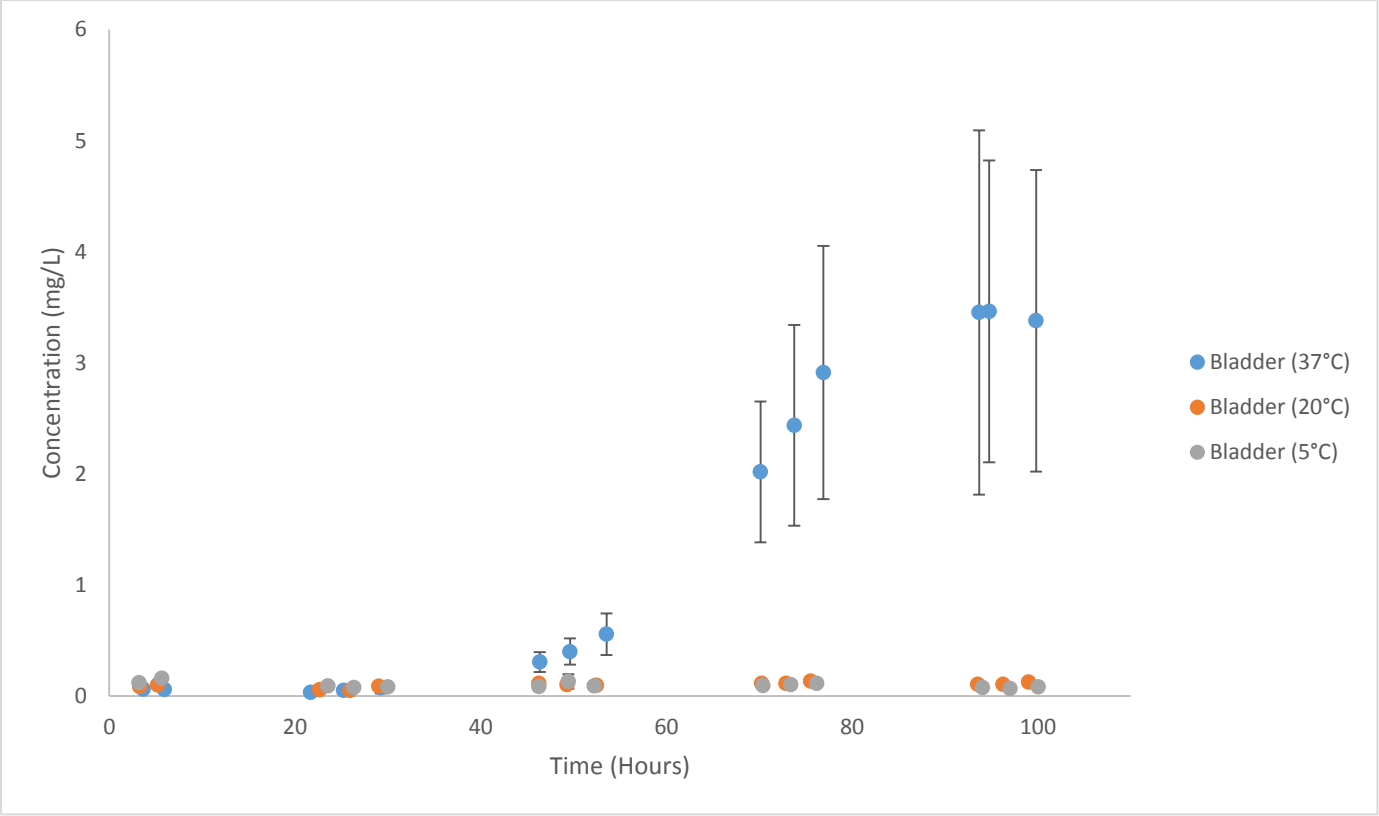

Figure 4.6 Average rhodamine B cumulative concentration with four sections from one bladder (intra-bladder sections), comparing bladder temperatures at 37, 20 and 5°C (pH 5) (n=4) (Error bars are ± S.D.)

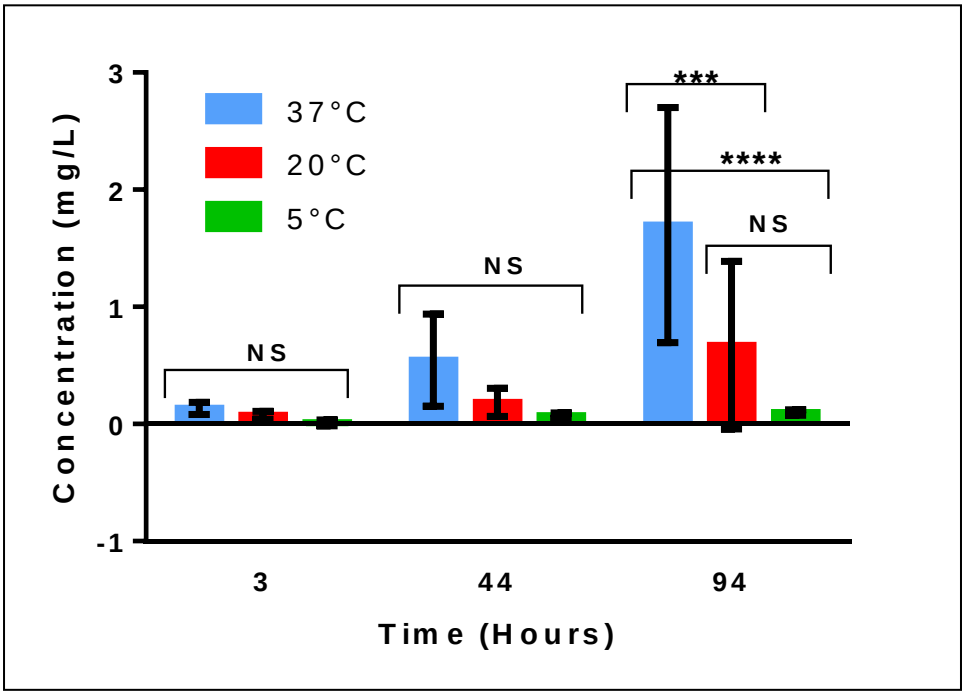

Figure 4.7 Two-Way ANOVA results of rhodamine B concentration in relation to the comparison of temperature (37, 20 and 5°C) using 100 mg/L rhodamine B with six fresh inter-bladder sections in pH 7.4 over approximately 100 hrs (Error bars are ± S.D)

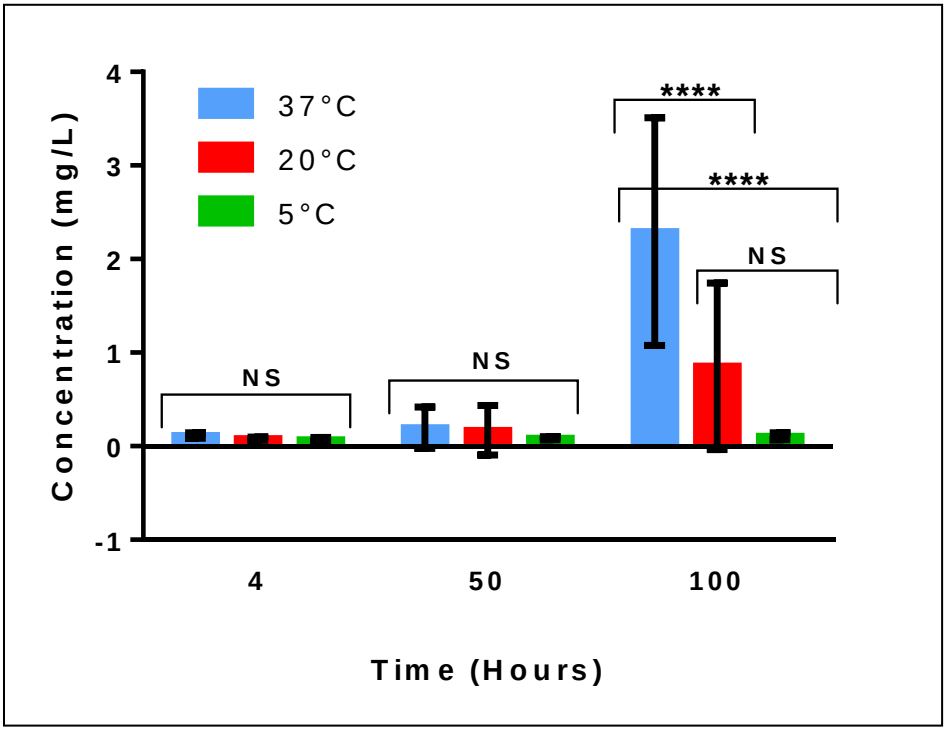

Figure 4.8 Two-Way ANOVA results of rhodamine B concentration in relation to the comparison of temperature (37, 20 and 5°C) using 100 mg/L rhodamine B with six fresh inter-bladder sections (pH 5) over approximately 100 hrs (Error bars are ± S.D.)

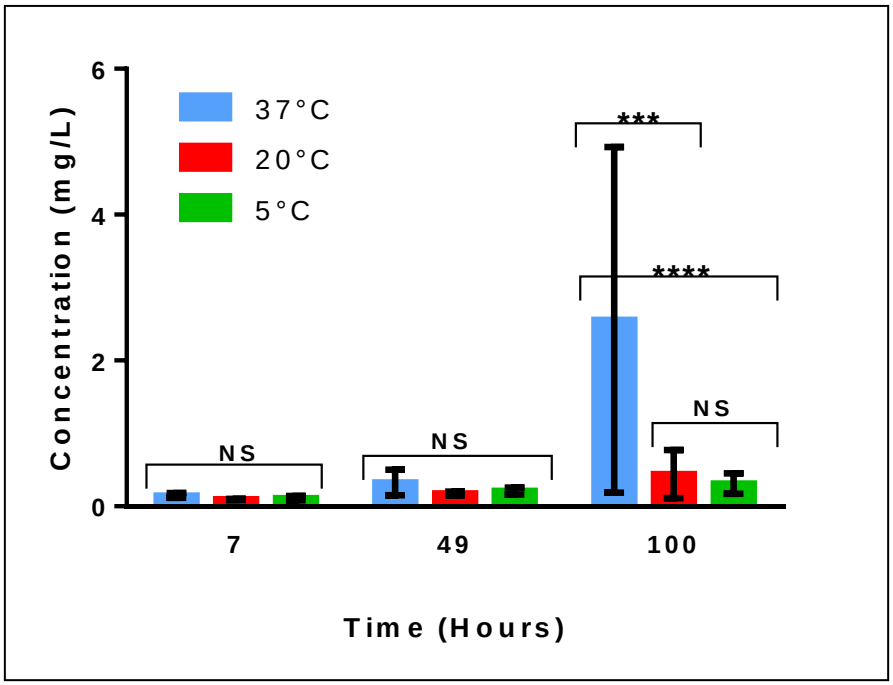

Figure 4.9 Two-Way ANOVA results of rhodamine B concentration in relation to the comparison of temperature (37, 20 and 5°C) using 100 mg/L rhodamine B with six degraded inter-bladder sections in pH 7.4 over approximately 100 hrs (Error bars are ± S.D.)

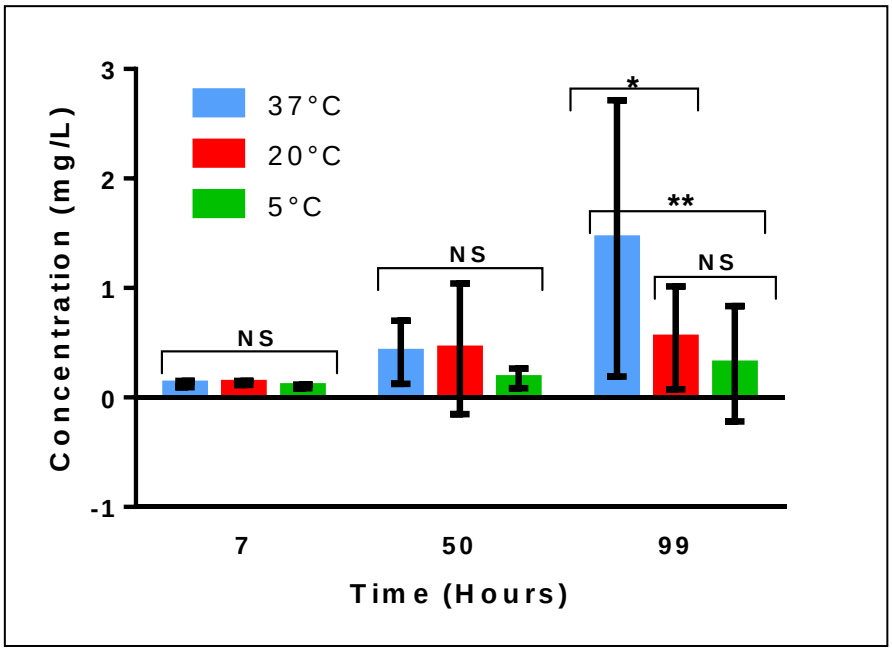

Figure 4.10 Two-Way ANOVA results of rhodamine B concentration in relation to the comparison of temperature (37, 20 and 5°C) using 100 mg/L rhodamine B with six degraded inter-bladder sections (pH 5) over approximately 100 hrs (Error bars are ± S.D.)

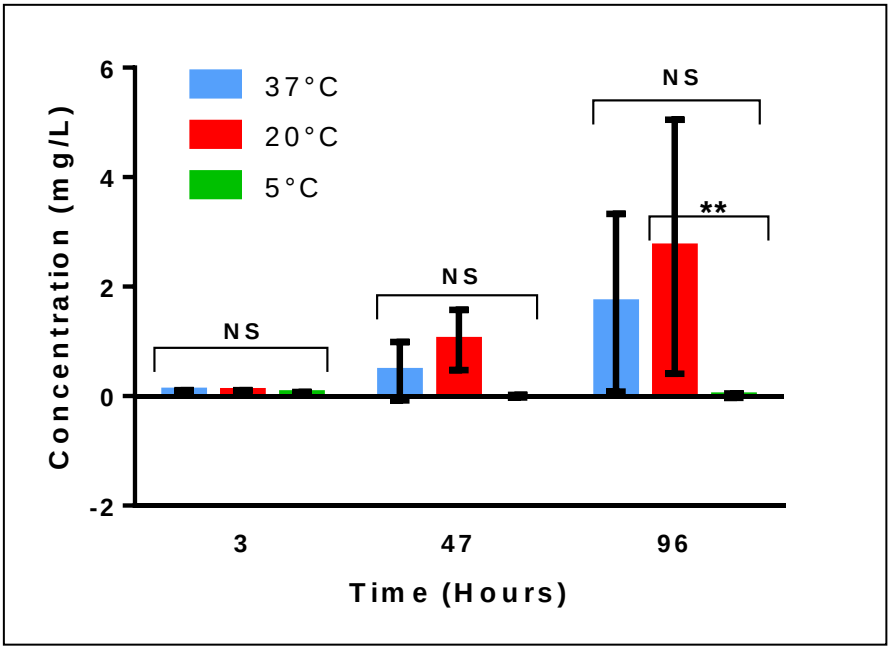

Figure 4.11 Two-Way ANOVA results of rhodamine B concentration in relation to the comparison of temperature (37, 20 and 5°C) using 100 mg/L rhodamine B with four sections from one fresh bladder (intra-bladder sections) in pH 7.4 over approximately 100 hrs (Error bars are ± S.D.)

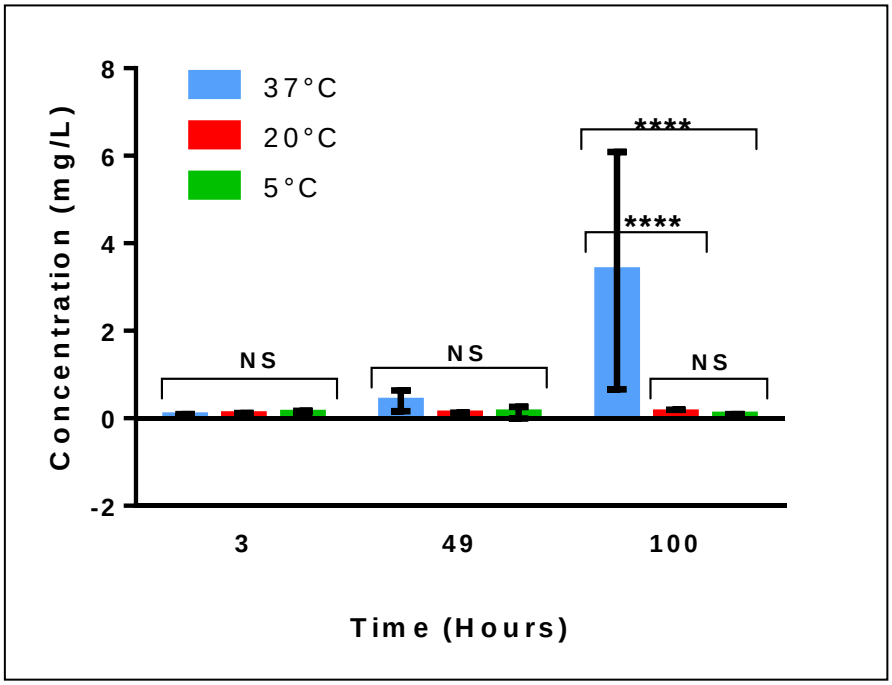

Figure 4.12 Two-Way ANOVA results of rhodamine B concentration in relation to the comparison of temperature (37, 20 and 5°C) using 100 mg/L rhodamine B with four sections from one fresh bladder (intra-bladder sections) (pH 5) over approximately 100 hrs (Error bars are ± S.D.)

4.3.1.2 amitriptyline and nortriptyline

The same parameters were used as for rhodamine B using three temperatures and two pH values to determine the effect on the diffusion of in this case two antidepressant drugs (See Section 4.3.1). A mixed solution of 100 mg/L amitriptyline and nortriptyline was the donor solution in two different pH solutions, PBS at pH 7.4 and AA at pH 5. Fresh inter-bladder sections were used in this set of experiments. Due to an interfering peak on the HPLC, the number of repeats was reduced. As a result, both drugs obtained results for all three temperatures at pH 7.4 however, at pH 5 there were only results at 37°C.

The results for amitriptyline at 37°C resulted in six samples from one of the bladder sections exceeding the top calibration (10 mg/L) from 70 hrs. As a result, these samples were removed from the graph as these samples were not diluted due to analysis of a number of experiments at once. The results for nortriptyline at 37°C in PBS had ten samples from three different bladder sections exceeding the 10 mg/L calibration limit therefore, these values were removed from the graphs as the concentration could not be reliably calculated.

4.3.1.2.1 Temperature comparison of the antidepressant drugs for the bladder sections

The pH 7.4 cumulative concentration graphs show the highest amitriptyline concentration was a single result of 6.69 mg/L at 37°C (See Figure 4.13). There were variations of amitriptyline within-day, which were not expected and could be due to the interfering peak as it did not interfere with all the samples (See Figure 4.13). The lowest cumulative amitriptyline concentration was in the pH 5 buffer solution at 5°C at 0.22 ± 0.03 mg/L (See Figure 4.15). The graph at pH 7.4 show the highest cumulative concentration of nortriptyline was also 6.69 ± 4.76 mg/L at 37°C (See Figure 4.14). The lowest cumulative nortriptyline concentration was 0.19 ± 0.01 in pH 7.4 buffer solution at 5°C (See Figure 4.14). The significant difference in the cumulative concentration of amitriptyline between the three temperatures was present at 100 ± 0.07 hrs at pH 7.4. The differences were observed between 37°C and 20°C (p 0.05) and also 37°C and 5°C ($p < 0.0001$). However, there was no difference between the two lower temperatures with similar cumulative concentrations at 20°C and 5°C (See Figure 4.17). There were not enough results to carry out an ANOVA for nortriptyline in PBS. Overall, this shows temperature would have a significant effect on the cumulative concentration of the two antidepressant drugs diffusing through bladder tissue over approximately 100 hrs after death.

4.3.1.2.2 pH comparison of the antidepressant drugs for the bladder sections

The pH seems to have an effect on the antidepressant drugs with no comparison at pH 5, as there were not enough results at 20°C and 5°C for a concentration graph. The concentration graphs of amitriptyline and nortriptyline at 37°C in pH 5 have been created (See Figures 4.15 and 4.16). The peak concentration of amitriptyline at 37°C (pH 5) was a single result of 1.96 mg/L. The peak concentration of nortriptyline at 37°C (pH 5) was 2.8 ± 0.32 mg/L. This shows lower concentrations of both drugs in comparison with pH 7.4. This is consistent with the ionisation state of both the drugs with ionisation states at 99% and 99.5% at pH 7.4 and 99.99% and 99.99% in pH 5. More of the drugs were unionised at the higher pH and this allowed for increased cumulative concentrations to diffuse through the bladder tissue.

4.3.1.2.3 PMI for significant differences in the cumulative concentrations of amitriptyline

The significant differences of the cumulative concentrations of amitriptyline between the temperatures only showed at the later stages of the experiment with the post-mortem interval of 100 ± 0.07 hrs. This shows the highest temperature (37°C) only show increased concentrations in comparison with the lowest temperatures (20°C and 5°C) after at least 4 days post-mortem (See Figure 4.17).

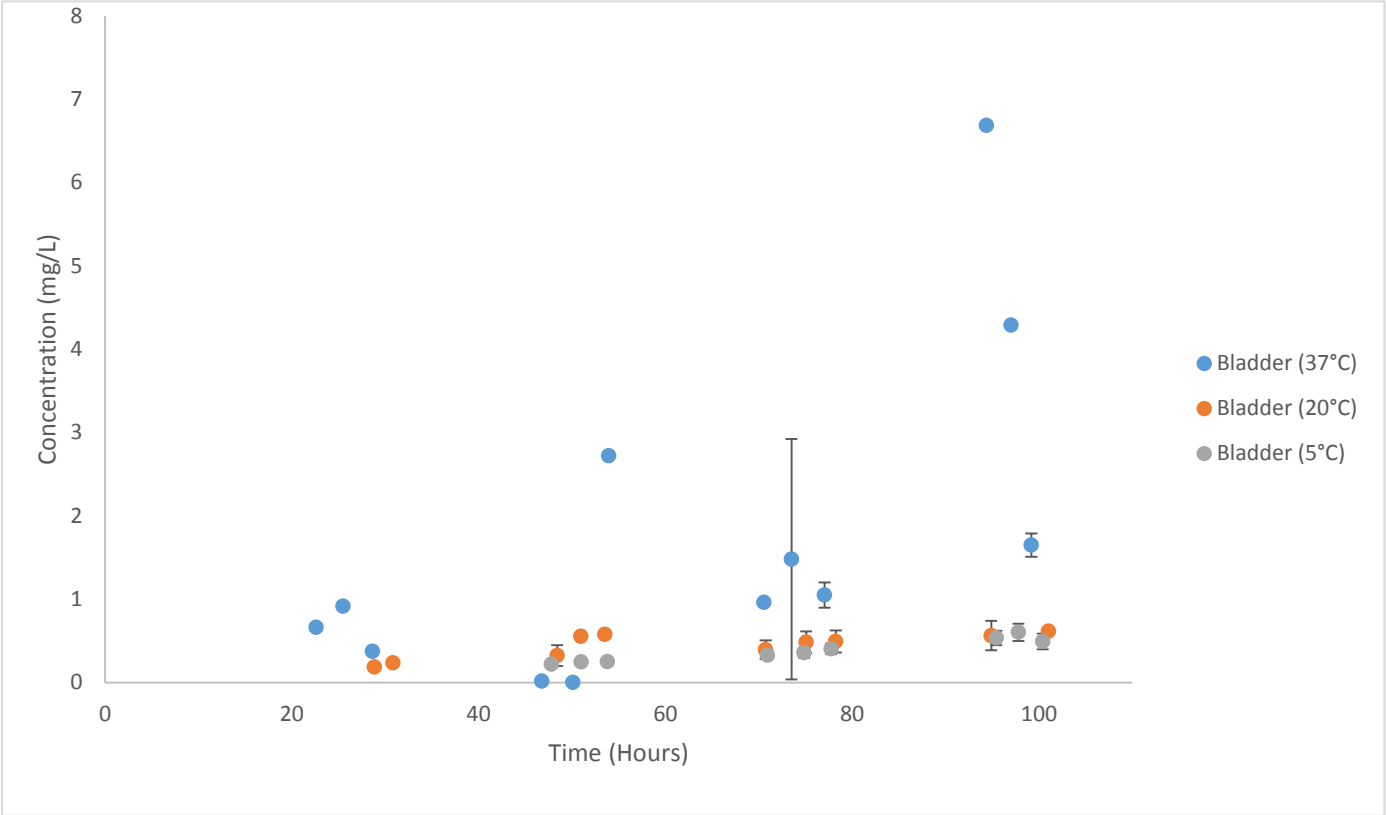

Figure 4.13 Average amitriptyline cumulative concentration with six fresh inter-bladder sections, comparing bladder temperatures at 37, 20 and 5°C in pH 7.4 (n=6) (Error bars are ± S.D.)

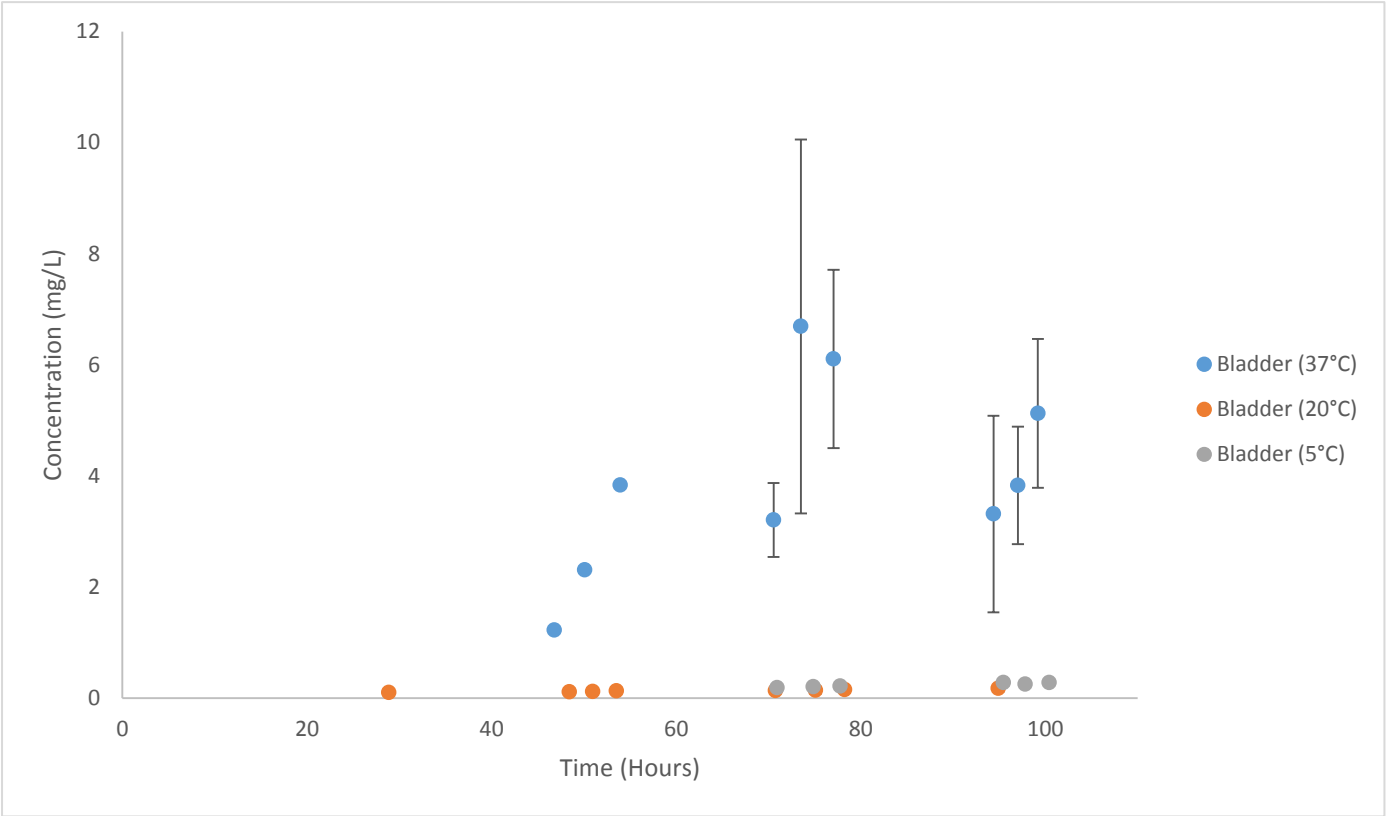

Figure 4.14 Average nortriptyline cumulative concentration with six fresh inter-bladder sections, comparing bladder temperatures at 37, 20 and 5°C in pH 7.4 (n=6) (Error bars are ± S.D.)

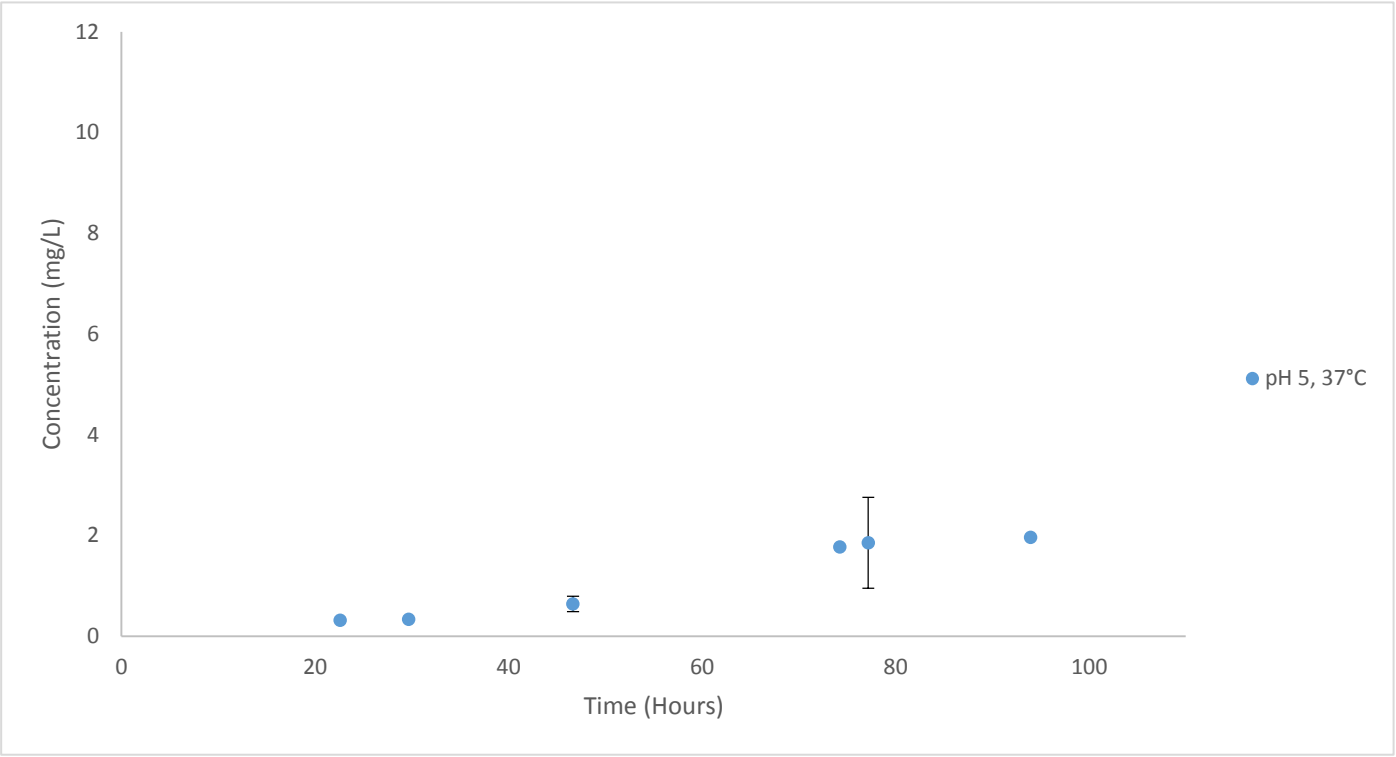

Figure 4.15 Average amitriptyline cumulative concentration with six fresh inter-bladder sections with a bladder temperatures at 37°C (pH 5) (n=6) (Error bars are ± S.D.)

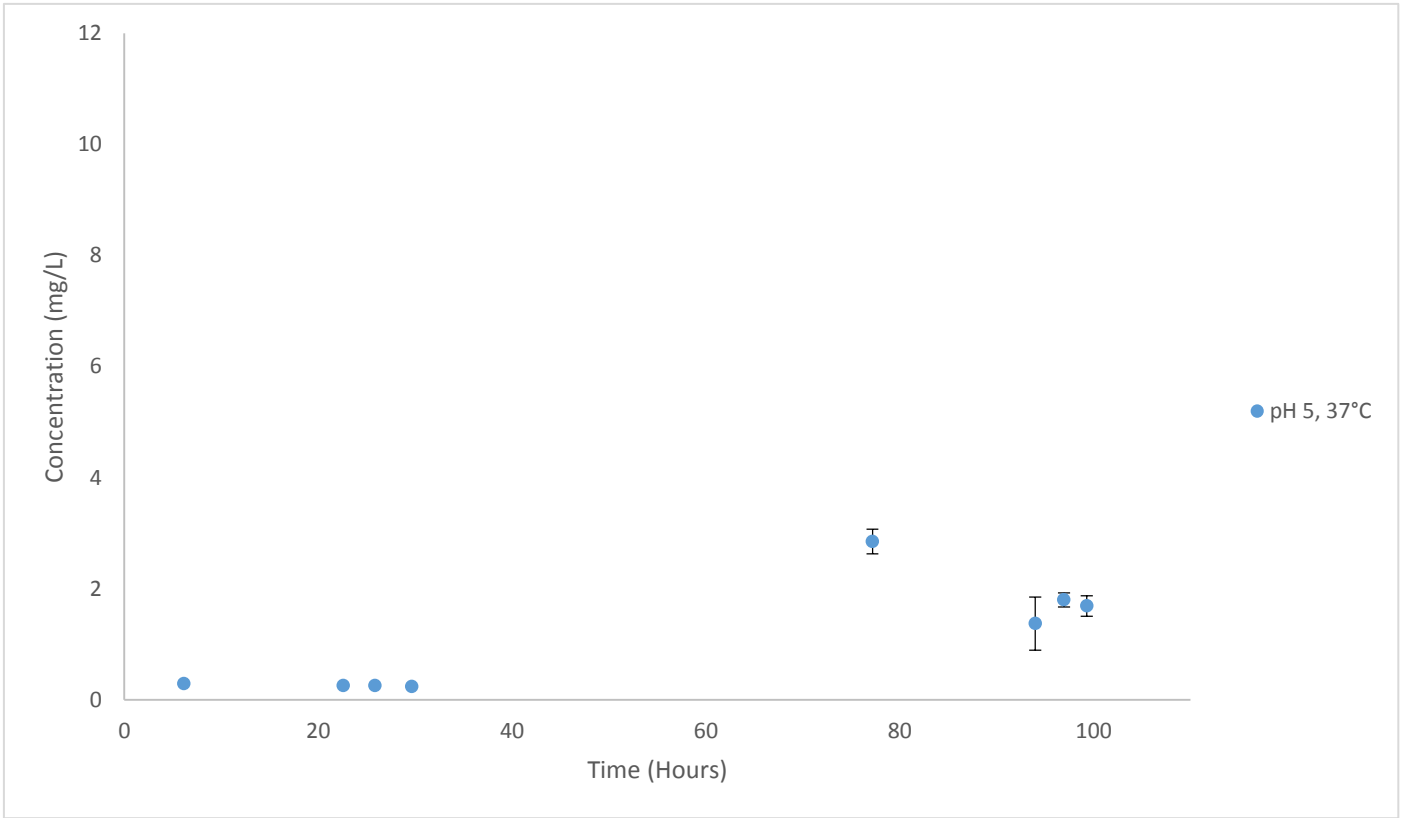

Figure 4.16 Average nortriptyline cumulative concentration with six fresh inter-bladder sections with a bladder temperatures at 37°C (pH 5) (n=6) (Error bars are ± S.D.)

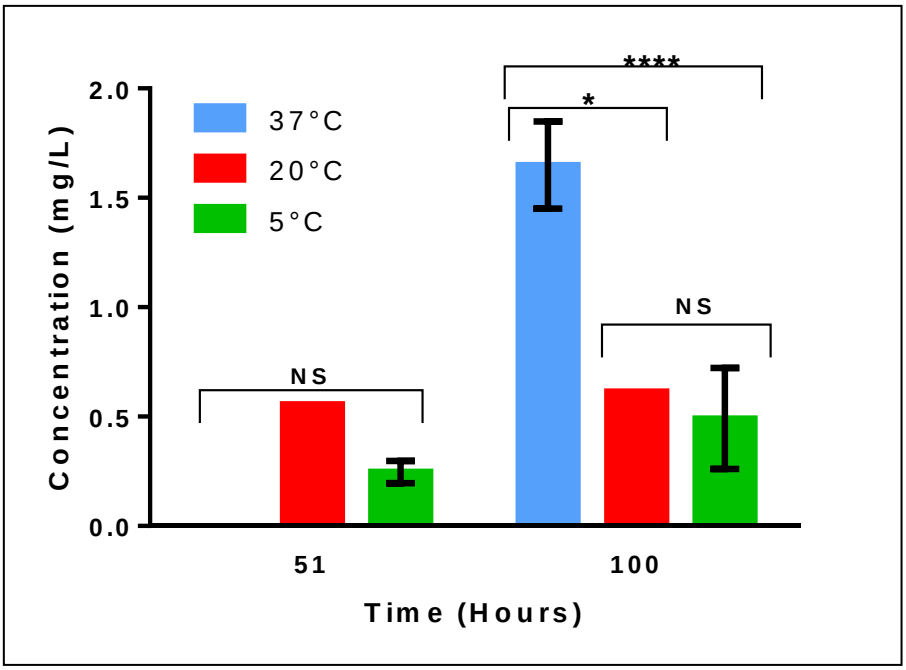

Figure 4.17 Two-Way ANOVA results of amitriptyline concentration in relation to the comparison of temperature (37, 20, and 5°C) using 100 mg/L amitriptyline with six fresh inter-bladder sections in pH 7.4 over approximately 100 hrs (Error bars are ± S.D.)

4.3.2 Influence of pH on the amount of drugs diffusing though bladder sections

The urine pH before and after death does not undergo much change with ranges of pH 5.0 – 8.5 and 4.6 – 8.5 respectively (Cook, Strauss and Caplan, 2007). However, the wide range of pH values could affect the drugs ionisation state. This is the reason for comparing two pH values, as both pH values can be found in the bladder. These values are compared alongside three temperatures (37, 20 and 5°C) using fresh and degraded bladder tissue.

4.3.2.1 Rhodamine B

Two solutions were used at two distinct pH values (pH 7.4 and pH 5) to compare the effect on the amount of diffusion of rhodamine B through bladder tissue. The conditions were the same as the temperature comparison in relation to rhodamine B comparison, bladder tissue condition and temperatures (See Section 4.3.1.1), as the same results were used to compare the result at the two pH values. The graphs show the highest cumulative concentration of rhodamine B was 3.46 ± 2.72 mg/L in pH 5 from the intra-bladders at 37°C (See Figure 4.24). The lowest cumulative rhodamine B concentration was from the fresh inter-bladder in pH 7.4 at 5°C, which was below the limit of detection of the rhodamine B method (See Figure 4.20).

4.3.2.1.1 pH comparison of rhodamine B for the bladder sections

The only significant difference in cumulative rhodamine B concentrations for the fresh inter-bladders was within the first 3 ± 0.12 hrs after death at 5°C with more diffusion from pH 5 (p 0.01) however, the concentrations were below the limit of detection from the rhodamine B method (See Figure 4.29). The rest of the fresh inter-bladder samples had no significant difference between the two pH values at all temperatures throughout the 100 hrs of experiments (See Figures 4.27 and 4.28). The peak cumulative rhodamine B concentrations for the fresh inter-bladders at the two different pH values are shown in table 4.1. The degraded inter-bladders showed no significant difference in cumulative rhodamine B concentration at any pH or temperature over the approximate 100 hrs (See Figures 4.30 - 4.32). The peak cumulative rhodamine B concentrations for the fresh inter-bladders at the two different pH values are shown in table 4.1. The fresh intra-bladder experiments resulted in two points of significant difference at 99 ± 0.04 hrs at 20°C with more diffusion from pH 7.4 (p 0.01) (See Figure 4.34) and 47 ± 0.14 hrs at 5°C with increased diffusion from pH 5 (p 0.05) (See Figure 4.35). There was no significant difference at any point at 37°C (See Figure 4.33). The peak cumulative rhodamine B concentrations for the fresh intra-bladders at the two different pH values are shown in table 4.1. Overall, this shows pH would not have a significant effect on the cumulative concentration of rhodamine B diffusing through bladder tissue over approximately 100 hrs after death.

4.3.2.1.2 Temperature comparison of rhodamine B for the bladder sections

The temperature has been shown to have an effect on rhodamine B diffusion for the fresh inter-bladders, as there is a steady increase in cumulative rhodamine B concentration over the course of approximately 100 hrs however the overall increase in concentration is less with the lower temperatures (See Figures 4.18 – 4.20). Similar results were observed for the degraded bladders, with an increase in cumulative rhodamine B concentration at 71 ± 0.08 hrs with higher concentrations at pH 7.4. The rhodamine B concentrations were

lower at the post-mortem temperatures (20°C and 5°C) than at the physiological temperature (See Figures 4.21 – 4.23). The intra-bladders showed a steady increase in cumulative rhodamine B concentrations over the course of the experiments at 37°C and 20°C however, only pH 7.4 increased at 20°C pH 5 showed no increase which was consistent with both pH at the lowest temperature (See Figures 4.24 – 4.26).

4.3.2.1.3 Other comparisons of rhodamine B for the bladder sections

Possible tissue degradation did not show to have an effect on the cumulative rhodamine B concentrations between the two pH and temperatures as both sets of cumulative concentration graphs resulted in similar behaviour of rhodamine B independent of fresh or degraded bladder tissue. However, one aspect was different where the fresh bladders had a lower cumulative concentration than the degraded bladders, which that was below the methods limit of detection (See Table 4.1).

The significant differences of the cumulative concentrations of rhodamine B between the temperatures was only present for the intra-bladder experiments with increased rhodamine B concentrations at 99 ± 0.04 hrs, 20°C in pH 7.4 (See Figure 4.34) and 47 ± 0.14 hrs at 5°C (pH 5) (See Figure 4.35). The rest of the experiments showed no significant differences in rhodamine B concentrations over the approximate 100 hrs (~4 days) post-mortem. However, the experiments show there is significant changes overtime.

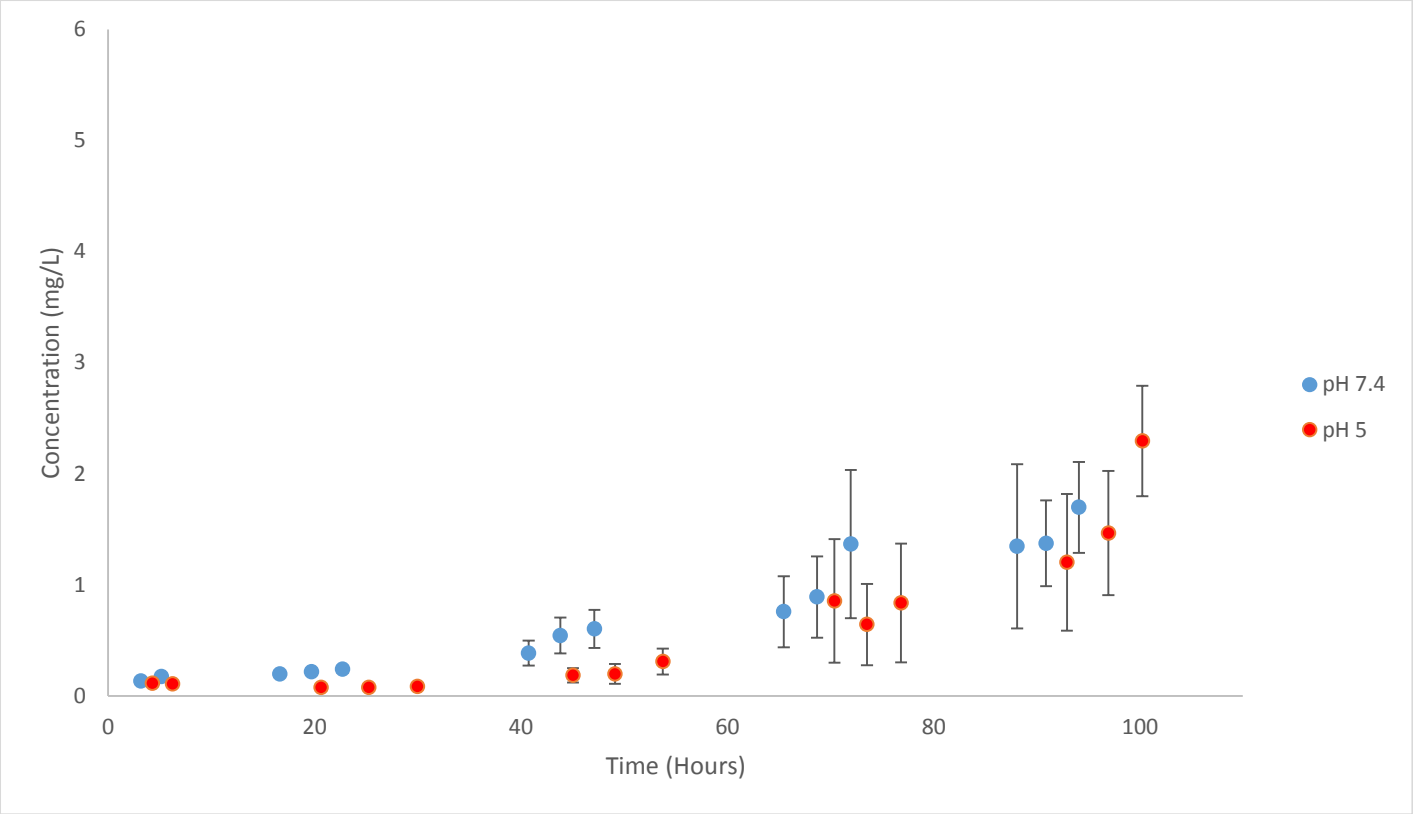

Figure 4.18 Average rhodamine B cumulative concentration with six fresh inter-bladder sections, comparing solution pH (pH 7.4 and 5) at 37°C (n=6) (Error bars are ± S.D.)

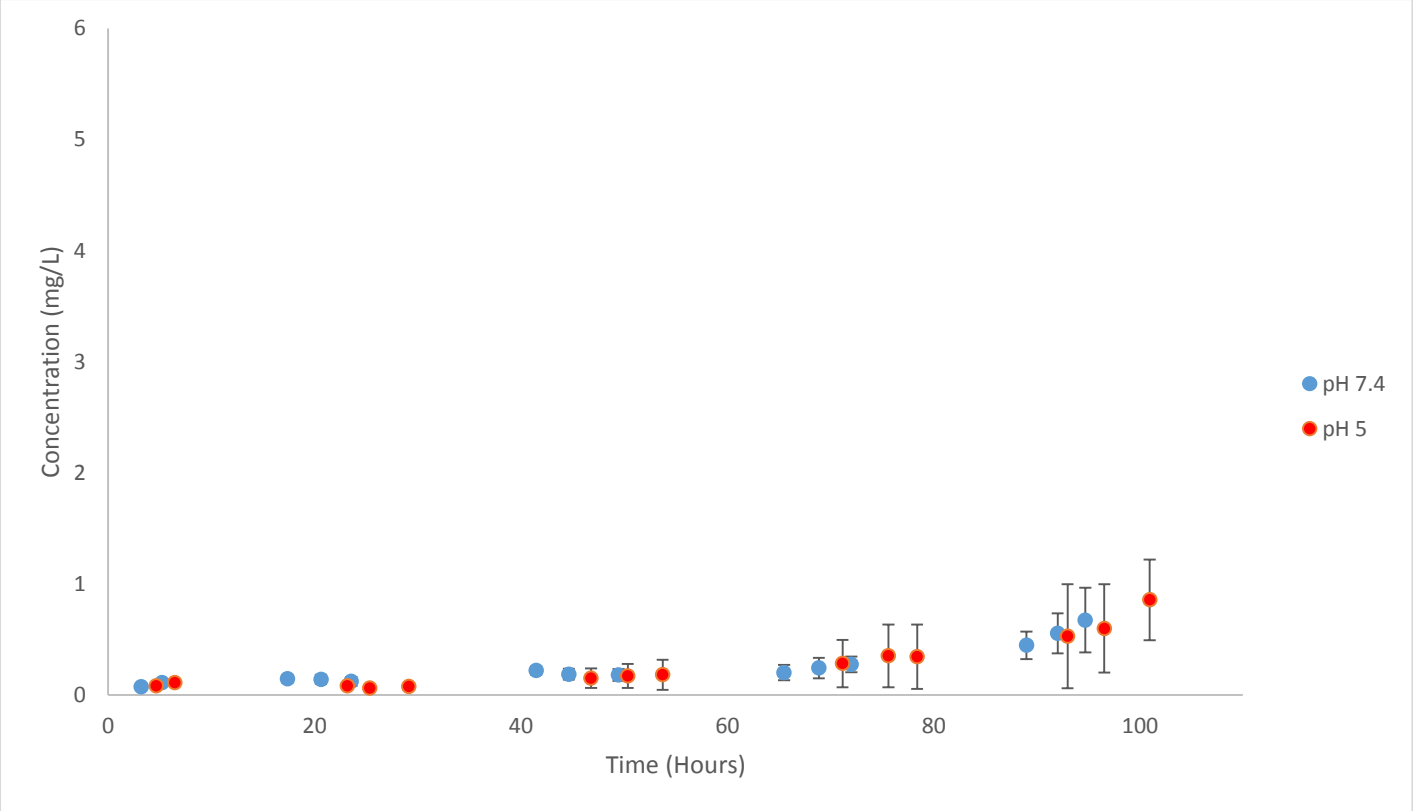

Figure 4.19 Average rhodamine B cumulative concentration with six fresh inter-bladder sections, comparing solution pH (pH 7.4 and 5) at 20°C (n=6) (Error bars are ± S.D.)

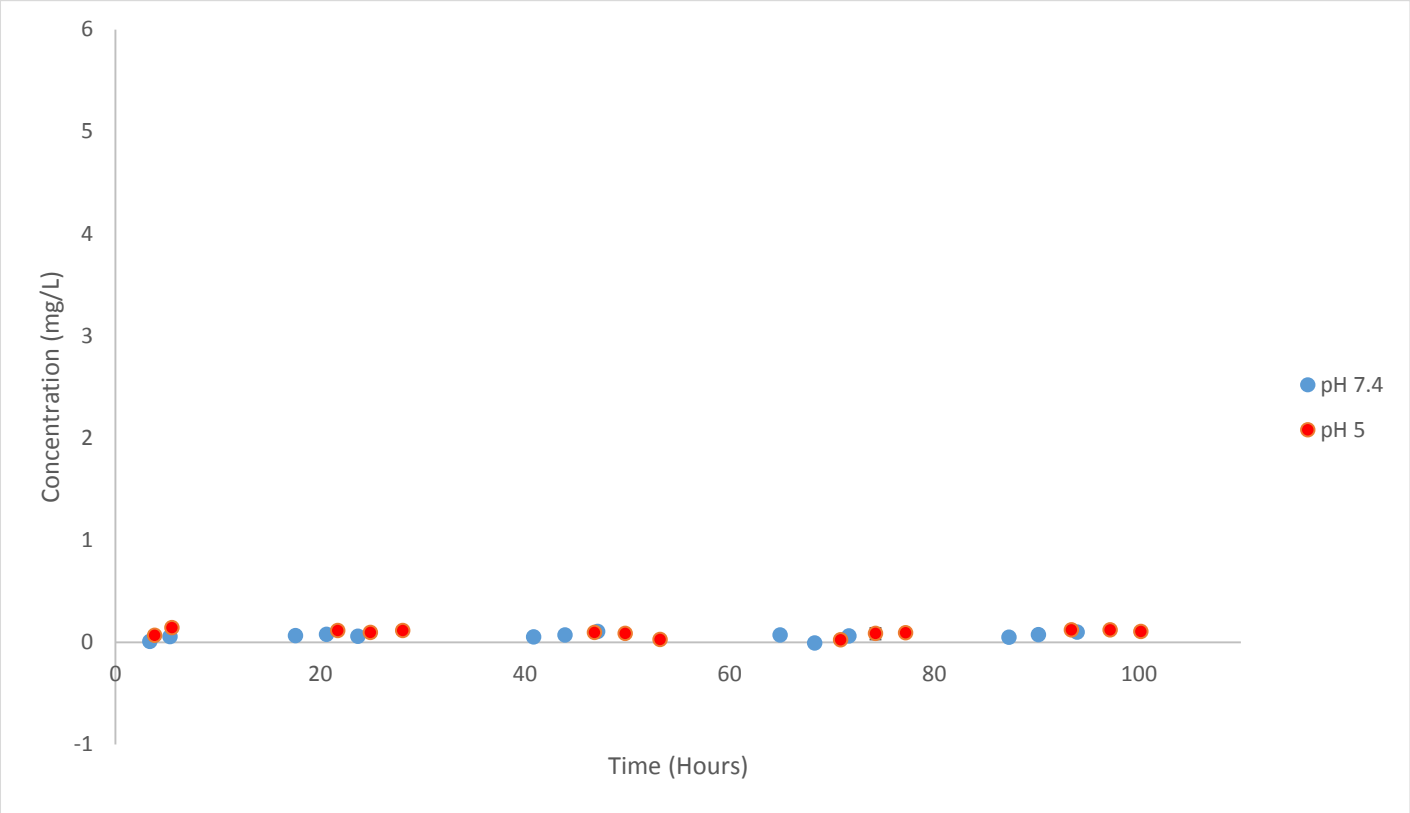

Figure 4.20 Average rhodamine B cumulative concentration with six fresh inter-bladder sections, comparing solution pH (pH 7.4 and 5) at 5°C (n=6) (Error bars are ± S.D.)

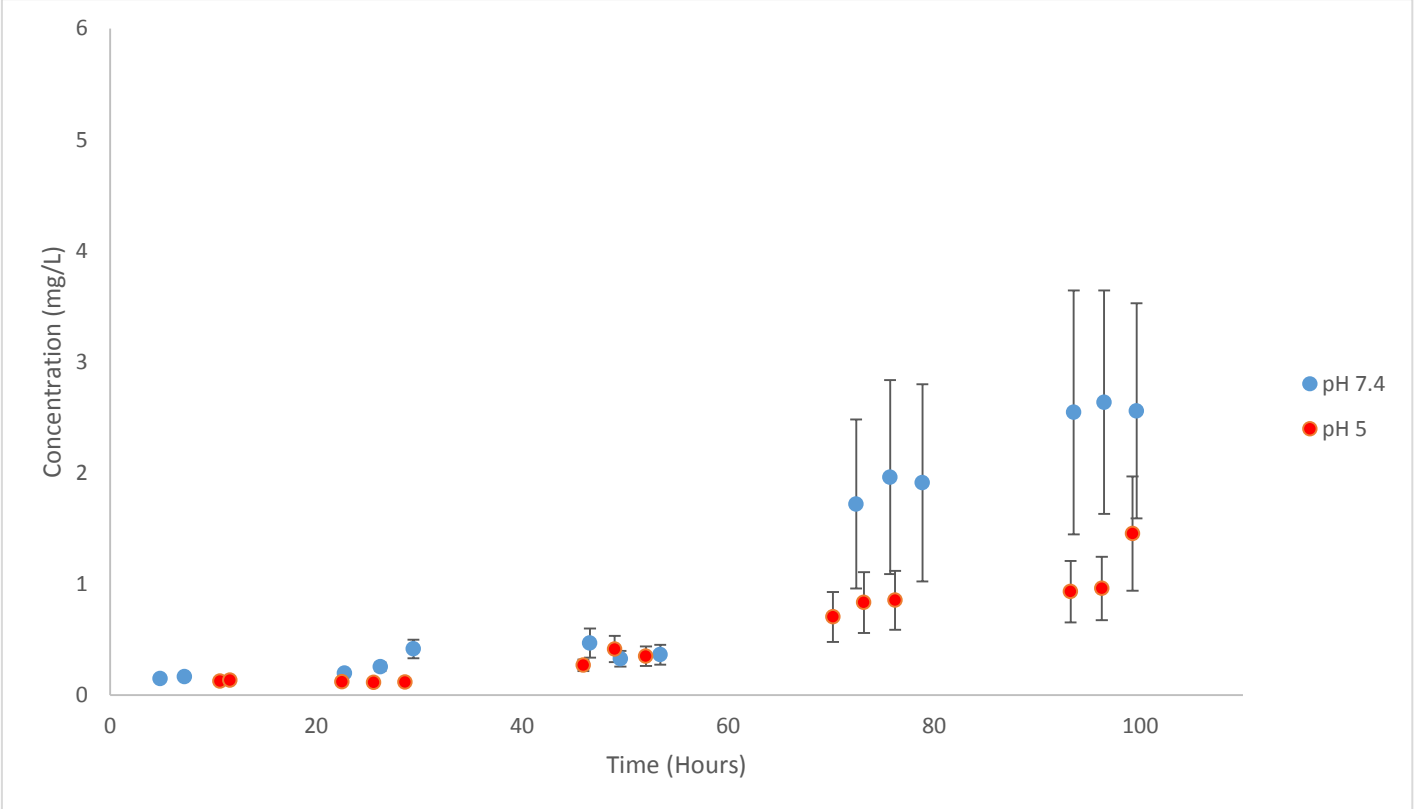

Figure 4.21 Average rhodamine B cumulative concentration with six degraded inter-bladder sections, comparing solution pH (pH 7.4 and 5) at 37°C (n=6) (Error bars are ± S.D.)

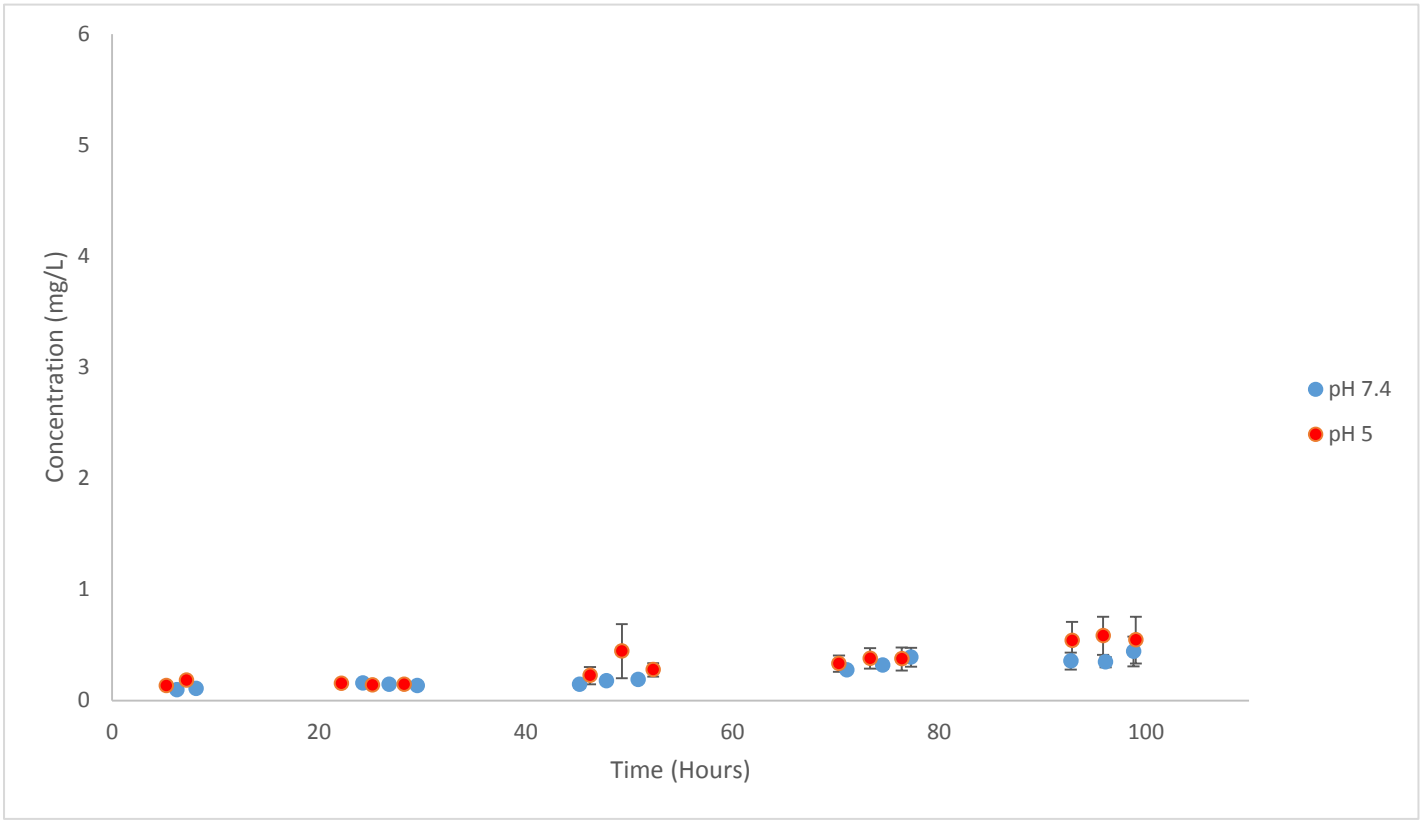

Figure 4.22 Average rhodamine B cumulative concentration with six degraded inter-bladder sections, comparing solution pH (pH 7.4 and 5) at 20°C (n=6) (Error bars are ± S.D.)

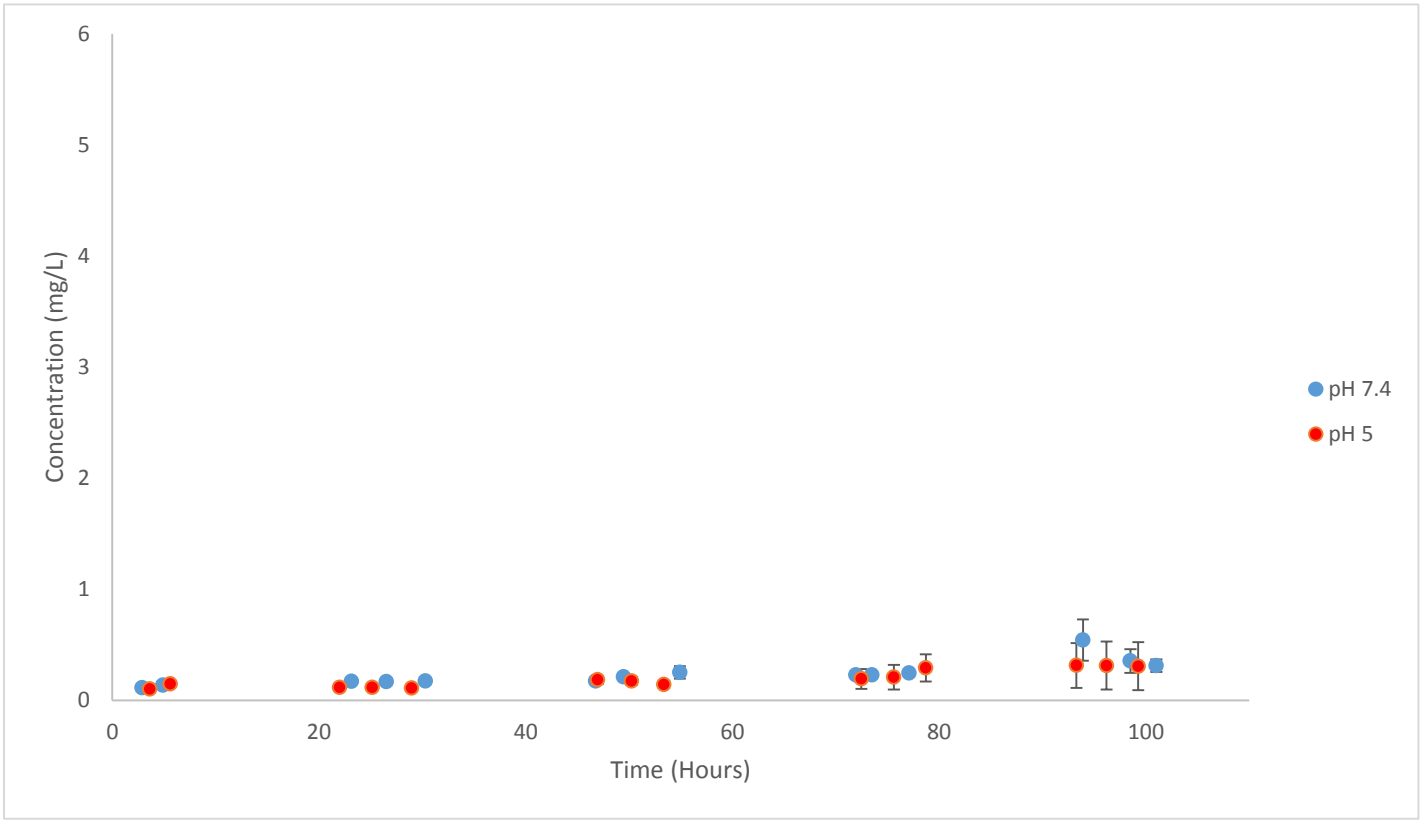

Figure 4.23 Average rhodamine B cumulative concentration with six degraded inter-bladder sections, comparing solution pH (pH 7.4 and 5) at 5°C (n=6) (Error bars are ± S.D.)

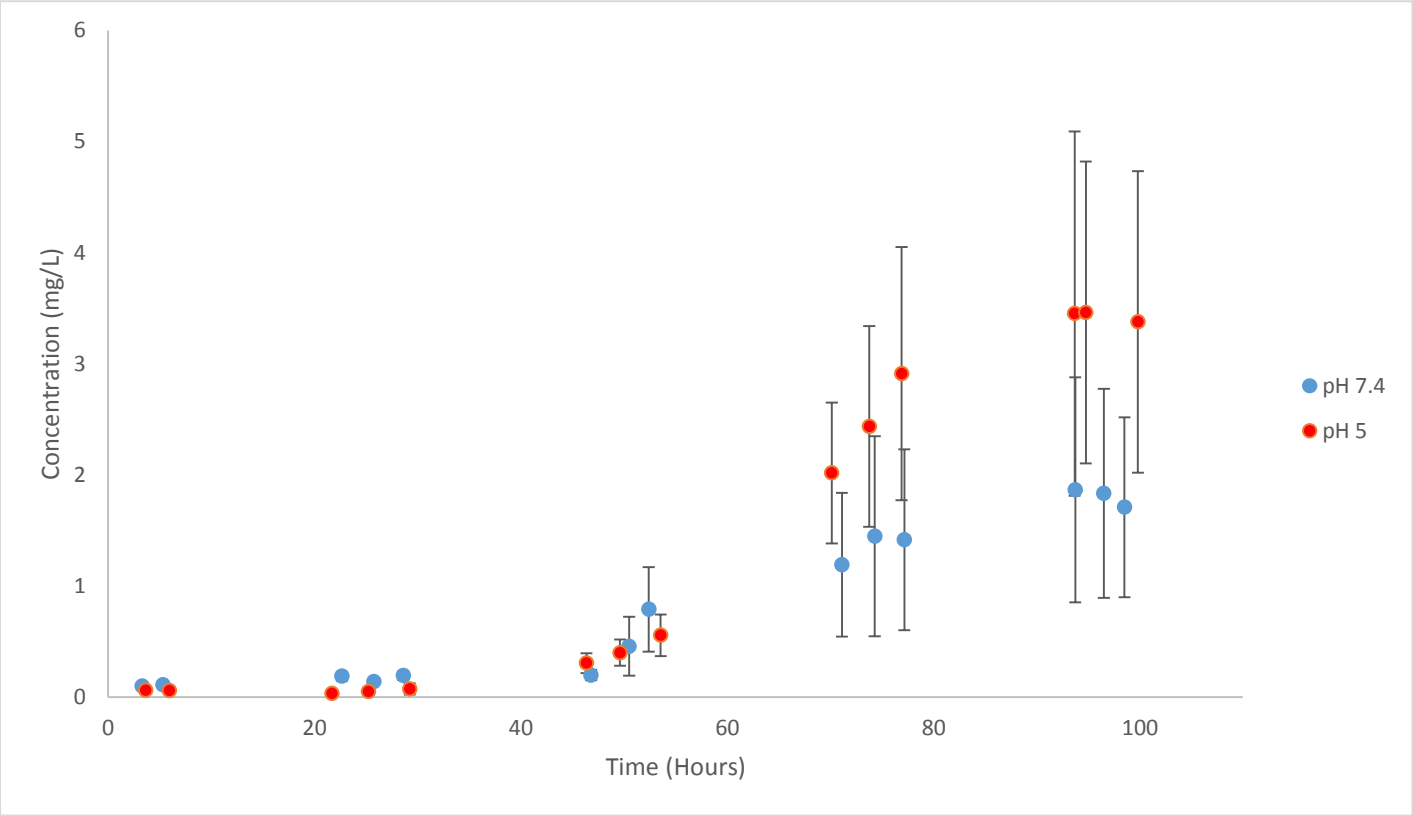

Figure 4.24 Average rhodamine B cumulative concentration with four sections from one fresh bladder (intra-bladder sections), comparing solution pH (pH 7.4 and 5) at 37°C (n=4) (Error bars are ± S.D.)

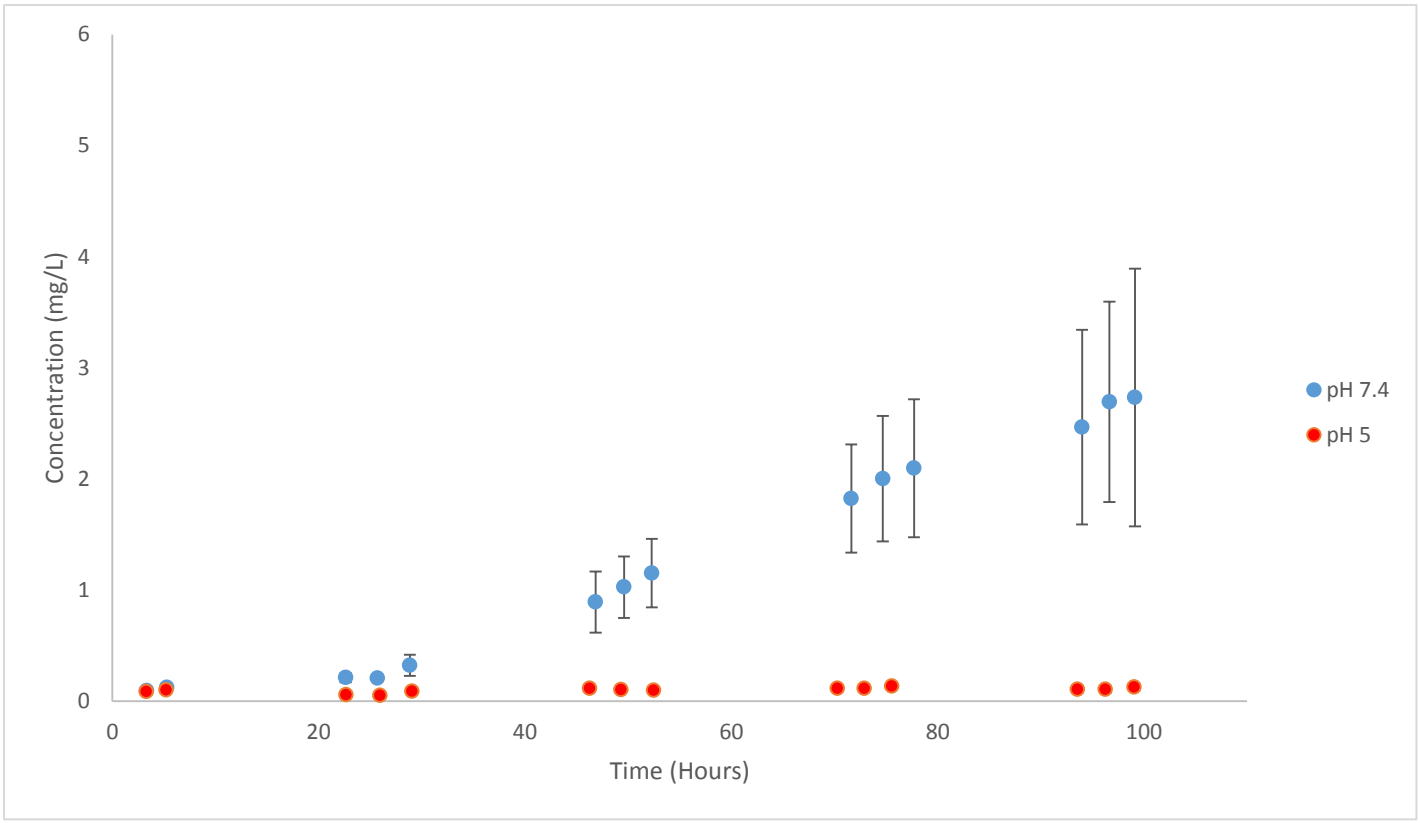

Figure 4.25 Average rhodamine B cumulative concentration with four sections from one fresh bladder (intra-bladder sections), comparing solution pH (pH 7.4 and 5) at 20°C (n=4) (Error bars are ± S.D.)

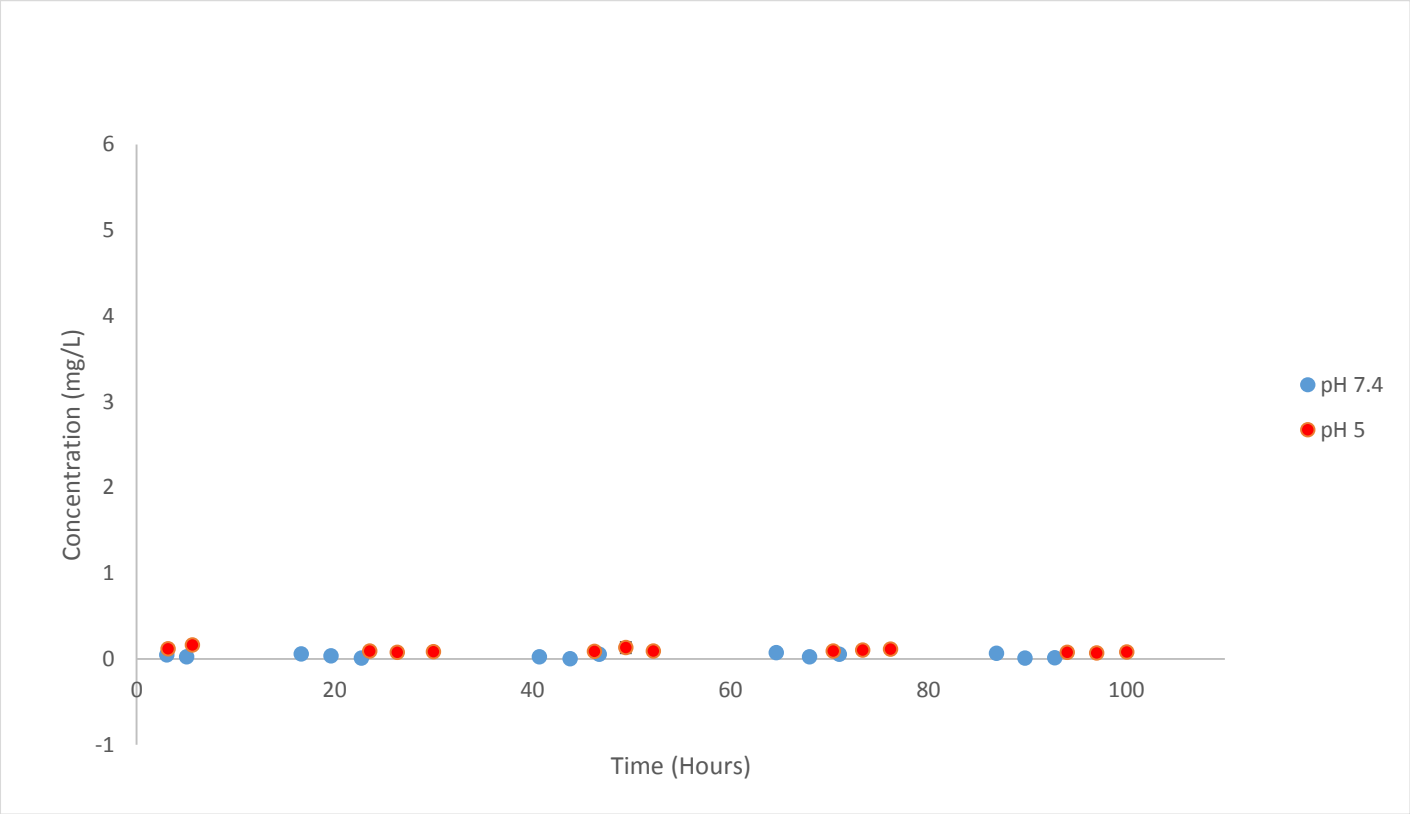

Figure 4.26 Average rhodamine B cumulative concentration with four sections from one fresh bladder (intra-bladder sections), comparing solution pH (pH 7.4 and 5) at 5°C (n=4) (Error bars are ± S.D.)

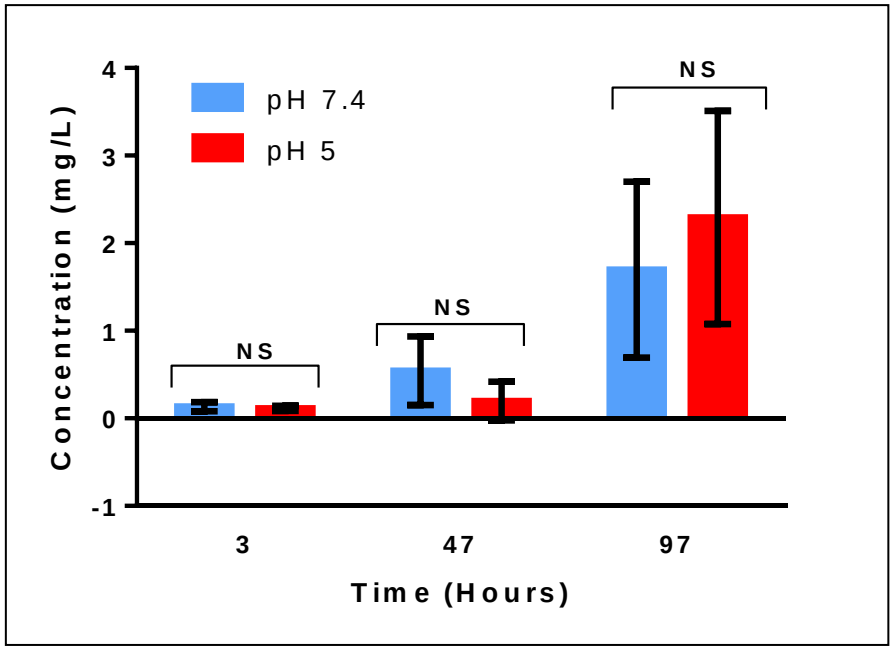

Figure 4.27 Two-Way ANOVA results of rhodamine B concentration in relation to the solution pH (pH 7.4 and 5) at 37°C using 100 mg/L rhodamine B with six fresh inter-bladder sections over approximately 100 hrs (Error bars are ± S.D.)

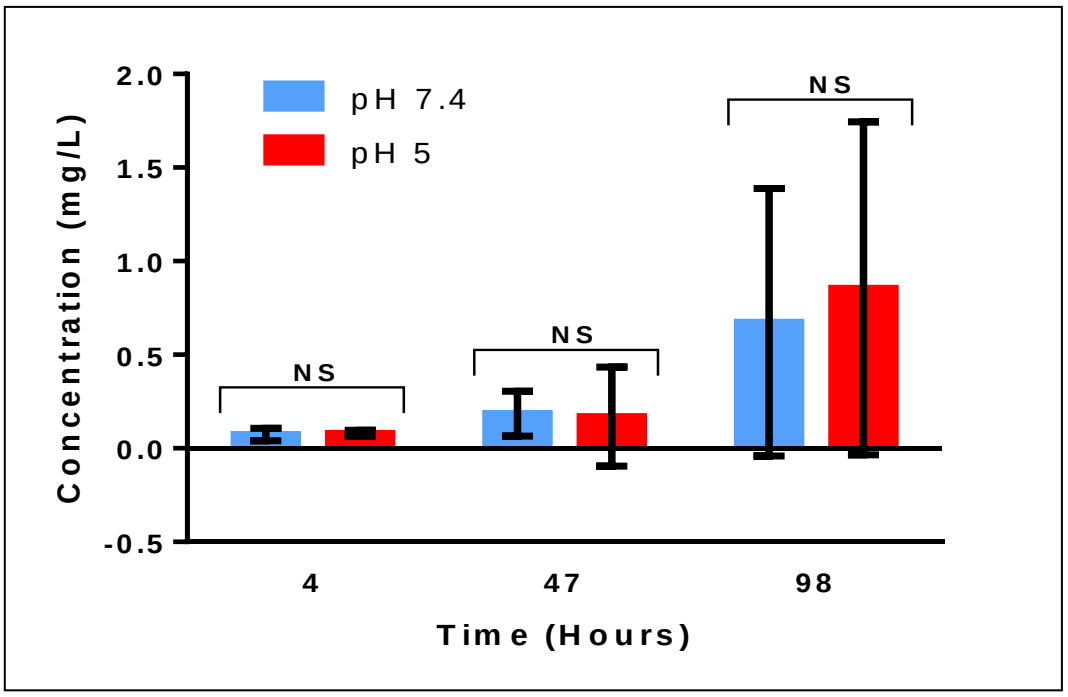

Figure 4.28 Two-Way ANOVA results of rhodamine B concentration in relation to the solution pH (pH 7.4 and 5) at 20°C using 100 mg/L rhodamine B with six fresh inter-bladder sections over approximately 100 hrs (Error bars are ± S.D.)

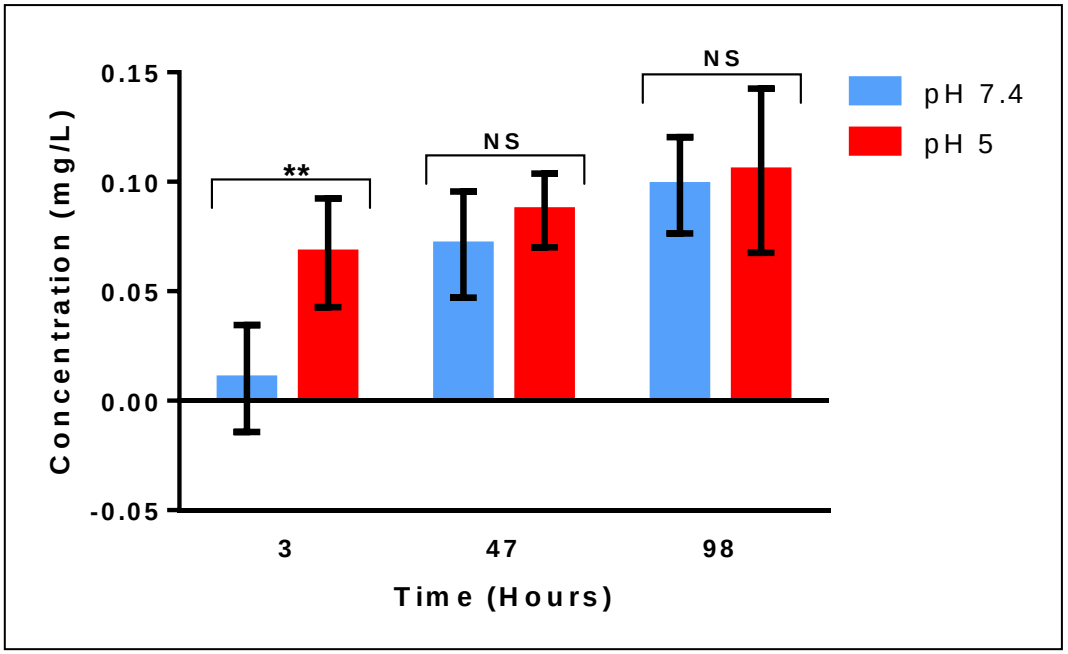

Figure 4.29 Two-Way ANOVA results of rhodamine B concentration in relation to the solution pH (pH 7.4 and 5) at 5°C using 100 mg/L rhodamine B with six fresh inter-bladder sections over approximately 100 hrs (Error bars are ± S.D.)

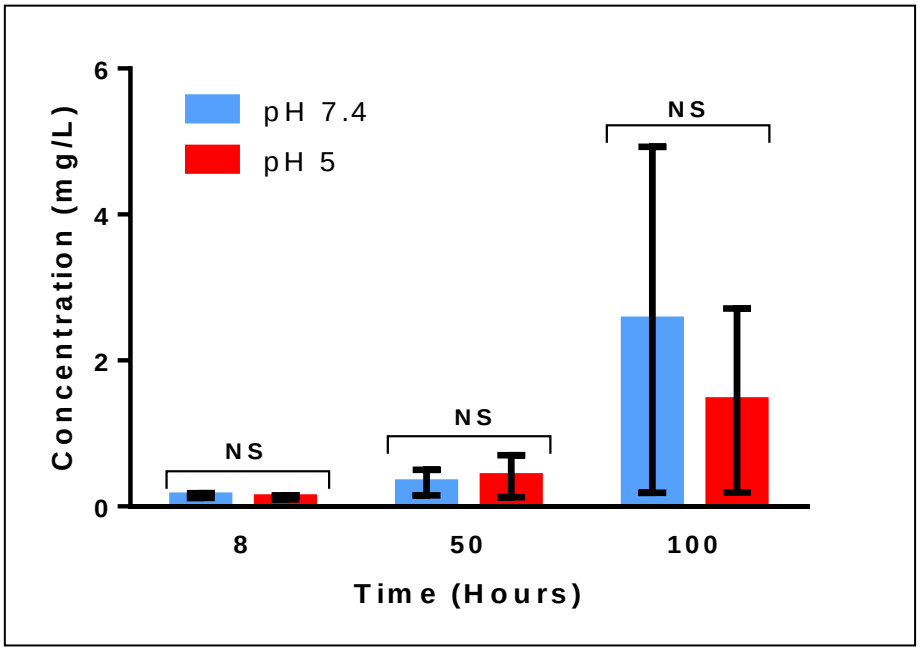

Figure 4.30 Two-Way ANOVA results of rhodamine B concentration in relation to the solution pH (pH 7.4 and 5) at 37°C using 100 mg/L rhodamine B with six degraded inter-bladder sections over approximately 100 hrs (Error bars are ± S.D.)

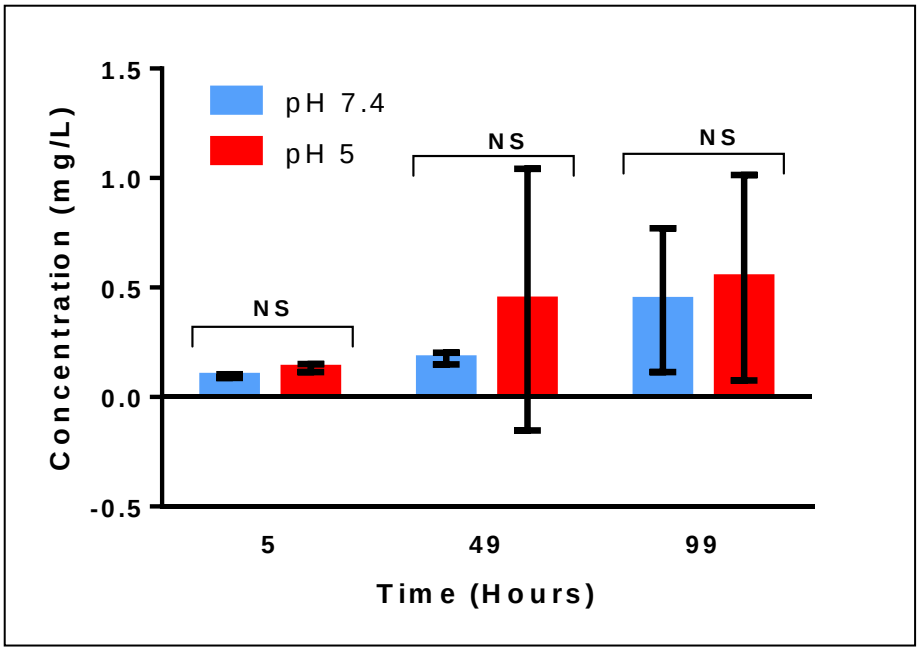

Figure 4.31 Two-Way ANOVA results of rhodamine B concentration in relation to the solution pH (pH 7.4 and 5) at 20°C using 100 mg/L rhodamine B with six degraded inter-bladder sections over approximately 100 hrs (Error bars are ± S.D.)

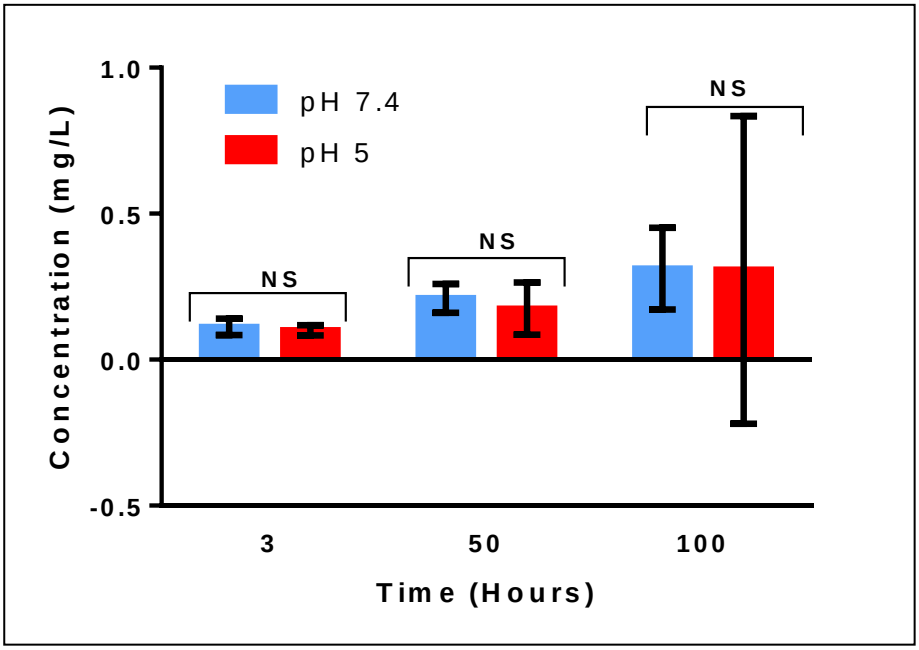

Figure 4.32 Two-Way ANOVA results of rhodamine B concentration in relation to the solution pH (pH 7.4 and 5) at 5°C using 100 mg/L rhodamine B with six degraded inter-bladder sections over approximately 100 hrs (Error bars are ± S.D.)

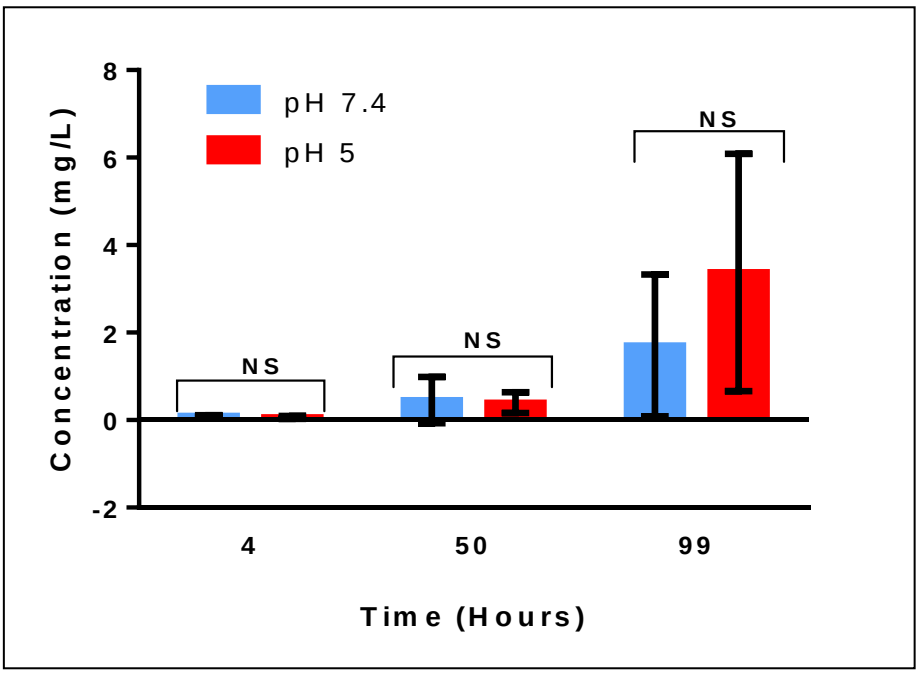

Figure 4.33 Two-Way ANOVA results of rhodamine B concentration in relation to the solution pH (pH 7.4 and 5) at 37°C using 100 mg/L rhodamine B with four sections from one fresh bladder (intra-bladder sections) over approximately 100 hrs (Error bars are ± S.D.)

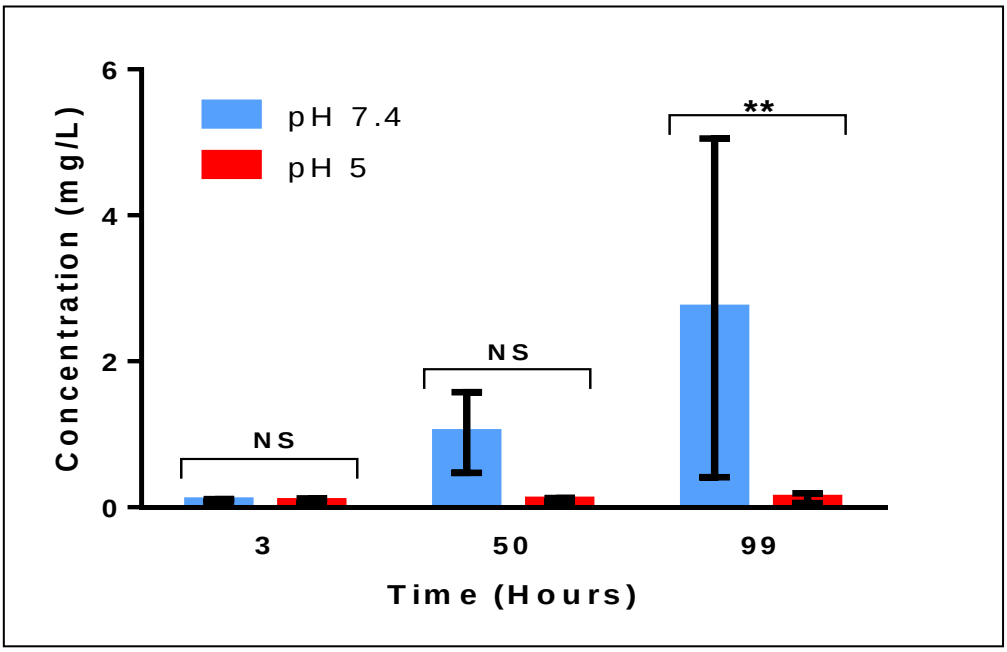

Figure 4.34 Two-Way ANOVA results of rhodamine B concentration in relation to the solution pH (pH 7.4 and 5) at 20°C using 100 mg/L rhodamine B with four sections from one fresh bladder (intra-bladder sections) over approximately 100 hrs (Error bars are ± S.D.)

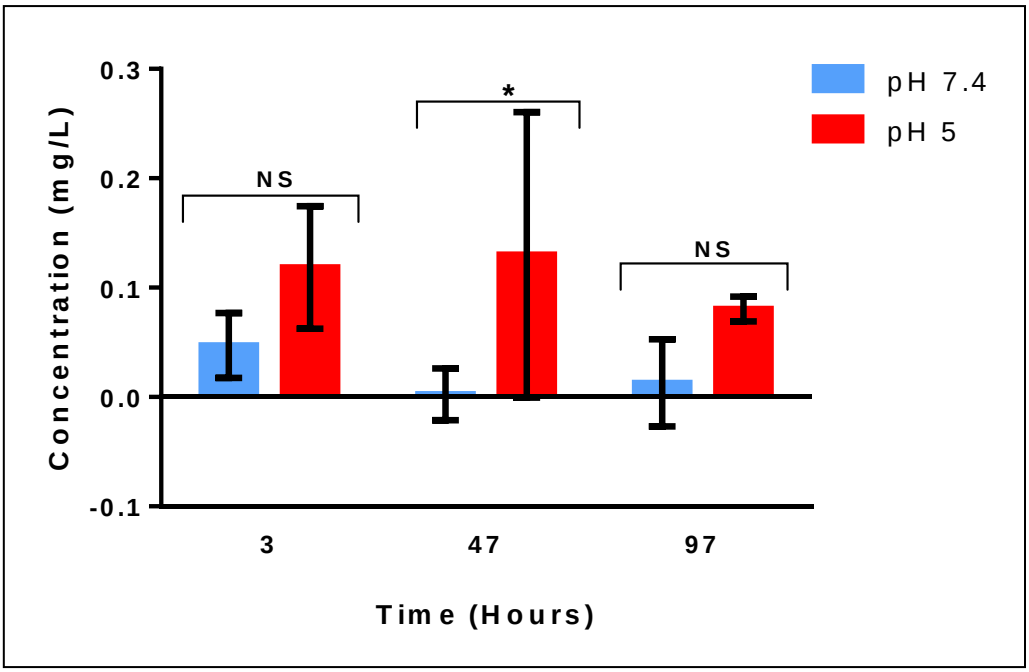

Figure 4.35 Two-Way ANOVA results of rhodamine B concentration in relation to the solution pH (pH 7.4 and 5) at 5°C using 100 mg/L rhodamine B with four sections from one fresh bladder (intra-bladder sections) over approximately 100 hrs (Error bars are ± S.D.)

4.3.2.2 amitriptyline and nortriptyline

The same conditions as previously stated with fresh inter-bladder sections used for a set of experiments at three temperatures (37°C, 20°C and 5°C) and two pH values (pH 7.4 and 5). These were used to determine the cumulative concentration of two antidepressant drugs diffusing through the bladder tissue during approximately 100 hrs. There were not enough results for a cumulative concentration graph at 20°C and 5°C in pH 5 buffer solution, therefore only results at 37°C were compared between the two buffer solutions.

The cumulative concentration graphs at 37°C show the highest cumulative concentration of amitriptyline was 6.68 mg/L from a single result (See Figure 4.36) and 6.69 ± 4.76 mg/L for nortriptyline both at pH 7.4 (See Figure 4.37). The lowest cumulative amitriptyline concentration was 0.31 ± 0.04 mg/L in AA buffer solution at pH 5 (See Figure 4.36) and 1.23 mg/L in PBS buffer solution at pH 7.4 from a single result for nortriptyline. Overall, this shows pH could have more of an effect on the cumulative concentration of amitriptyline than nortriptyline diffusing through bladder tissue over approximately 100 hrs after death.

There is a more sporadic increase in the cumulative concentration of both antidepressant drugs at physiological conditions (37°C) and pH 7.4 buffer solution, also there was lower concentration increase at pH 5. The difference between the cumulative concentrations of both antidepressants showed no significant difference between the pH for amitriptyline (p 0.06) and nortriptyline (p 0.15). This could be due to the limited number of available results to compare. An increased number of results may give a more accurate result. Overall, these results show that pH is not a significant factor in affecting the rate of diffusion of amitriptyline and nortriptyline up to 100 hrs after death.

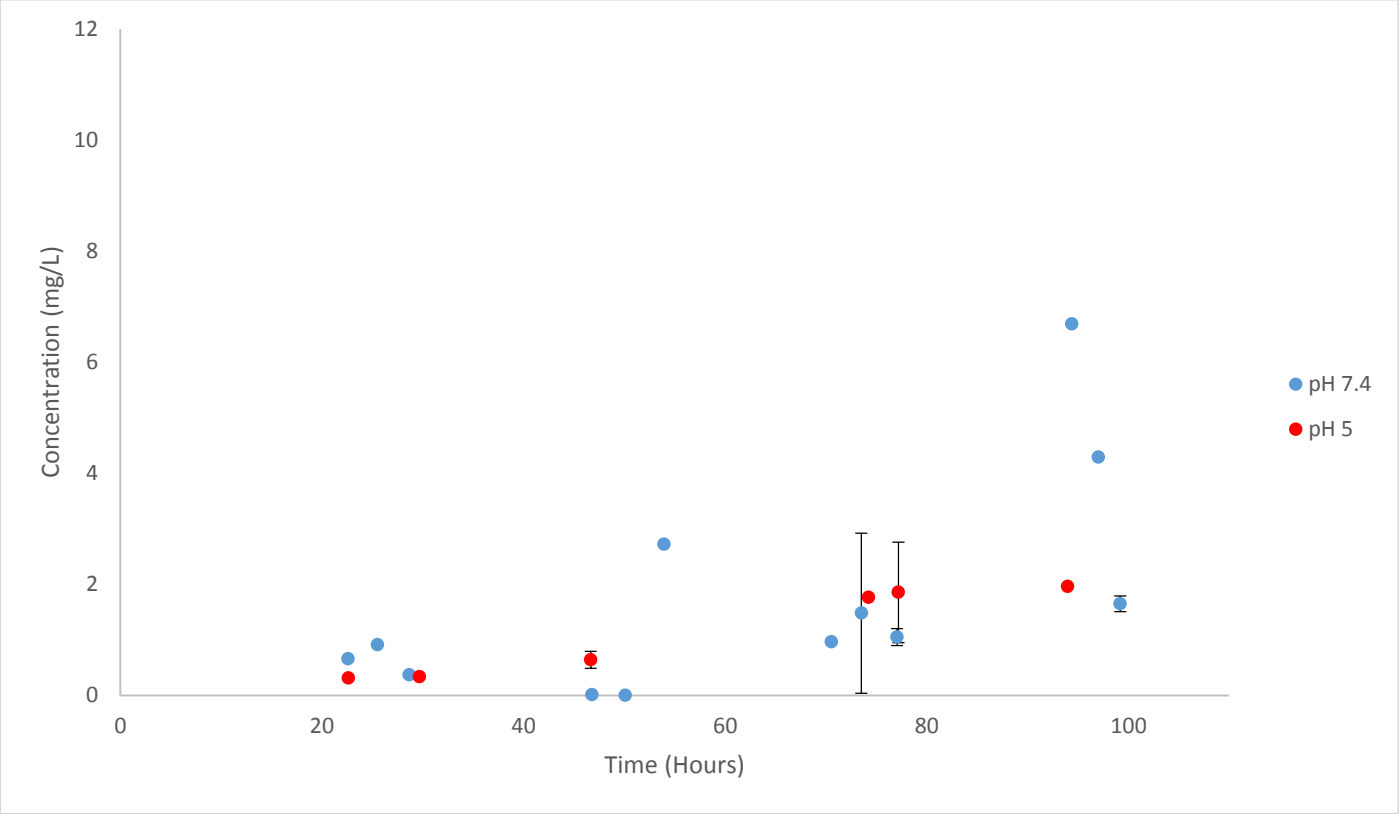

Figure 4.36 Average amitriptyline cumulative concentration with six fresh inter-bladder sections, comparing solution pH (pH 7.4 and 5) at 37°C (n=6) (Error bars are ± S.D.)

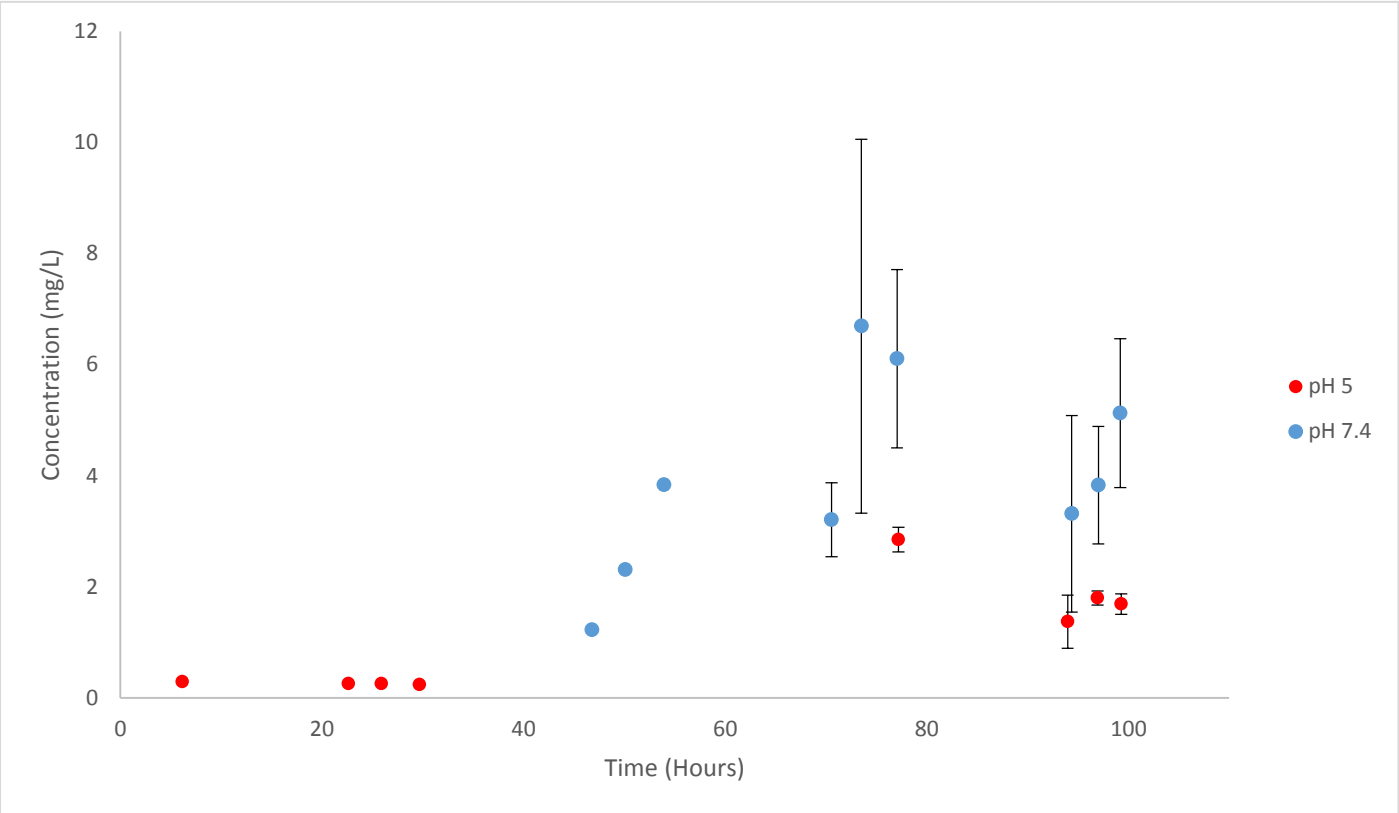

Figure 4.37 Average nortriptyline cumulative concentration with six fresh inter-bladder sections, comparing solution pH (pH 7.4 and 5) at 37°C (n=6) (Error bars are ± S.D.)

4.3.3 Influence of degradation on the amount of rhodamine B diffusing through bladder sections

The decomposition process begins within minutes after death changing every cell within the body and as a result the body beings to break down. This has been suggested as one of the reasons for the movement of drugs within the body after death. This would be especially true in the bladder where any holes would allow urine to leak into the peritoneal cavity. This suggests that post-mortem toxicology samples taken closer to the time of death would result in values that were closer to drug concentrations at the time of death as the drugs would not have had chance to diffuse or move around the body. As a result, the longer the post-mortem interval, the more tissue degradation increasing the amount of drugs released changing the drug concentrations around the body. Specifically, tissue degradation could affect the concentration of drugs released into the peritoneal cavity from the bladder. In order to study the possible degradation of the bladder the experiments were repeated using bladder tissue that had been thawed after collection and being frozen for one week prior to the analysis. The same conditions as the previous experiments were used (100 mg/L rhodamine B in the donor chamber, temperature (37, 20, and 5°C) and pH (pH 7.4 and 5)). The results show the highest cumulative concentration of rhodamine B was 2.63 ± 1.01 mg/L from the frozen inter-bladders in pH 7.4 at 37°C (See Figure 4.38). The lowest cumulative rhodamine B concentration was from the fresh inter-bladder in pH 7.4 at 5°C, which was below the limit of detection of the rhodamine B method (See Figure 4.40).

4.3.3.1 Tissue degradation comparison of rhodamine B for the bladder sections

The only significant difference in cumulative rhodamine B concentrations was increased concentration of rhodamine B from the degraded bladders in pH 7.4 buffer solution at 5°C at all time points, 3 ± 0.12 hrs (p 0.05), 47 ± 0.09 hrs (p 0.01) and 98 ± 0.06 hrs (p 0.0001) (See Figure 4.46). There was no significant difference between fresh and degraded bladder tissue at 37°C and 20°C at pH 7.4 throughout the first 100 hrs after death (See Figures 4.44 and 4.45). There was no significant difference between fresh and degraded bladder tissue in AA buffer solution at pH 5 at any of the three temperatures (See Figures 4.47 – 4.49). The peak cumulative rhodamine B concentrations for the fresh and degraded bladder tissues are shown in table 4.1. Overall, this shows degradation would not have a significant effect on the cumulative concentration of rhodamine B diffusing through previously frozen bladder tissue over approximately 100 hrs after death.

4.3.3.2 Temperature comparison of rhodamine B for the bladder sections

The tissue degradation was investigated at three different temperatures and this factor seems to affect the concentrations of rhodamine B diffusing through the bladder tissue. The highest temperature (37°C) shows increased concentration of rhodamine B rising steadily throughout the experiment in PBS buffer solution at pH 7.4 (See Figure 4.38). The two lower temperatures do not show an increase in rhodamine B concentration over the course of the experiment at pH 7.4 (See Figures 4.39 and 4.40). Similar results were observed in AA buffer solution at pH 5 for the three temperatures with 37°C resulting in higher concentrations of rhodamine B than the lower concentrations (See Figures 4.41 – 4.43).

4.3.3.3 pH comparison of rhodamine B for the bladder sections

The different buffer solutions used to compare two pH values show there is a difference between the peak rhodamine B concentrations at 37°C with more diffusion from pH 7.4, with peak concentrations at 2.63 ± 2.46 mg/L (pH 7.4) and 1.45 ± 1.26 mg/L (pH 5) (See Table 4.1). However, due to the large standard deviation the overlap in concentrations show the difference is not significant.

4.3.3.4 PMI for significant differences in the cumulative concentrations of rhodamine B

The significant differences of the cumulative concentrations of rhodamine B between the temperatures was only present for the inter-bladder experiment in pH 7.4 at 5°C from 3 ± 0.12 hrs with the degraded bladder tissue showing increased rhodamine B concentrations (See Figure 4.46). The rest of the experiments showed no significant differences in rhodamine B concentrations over the 100 hrs post-mortem.

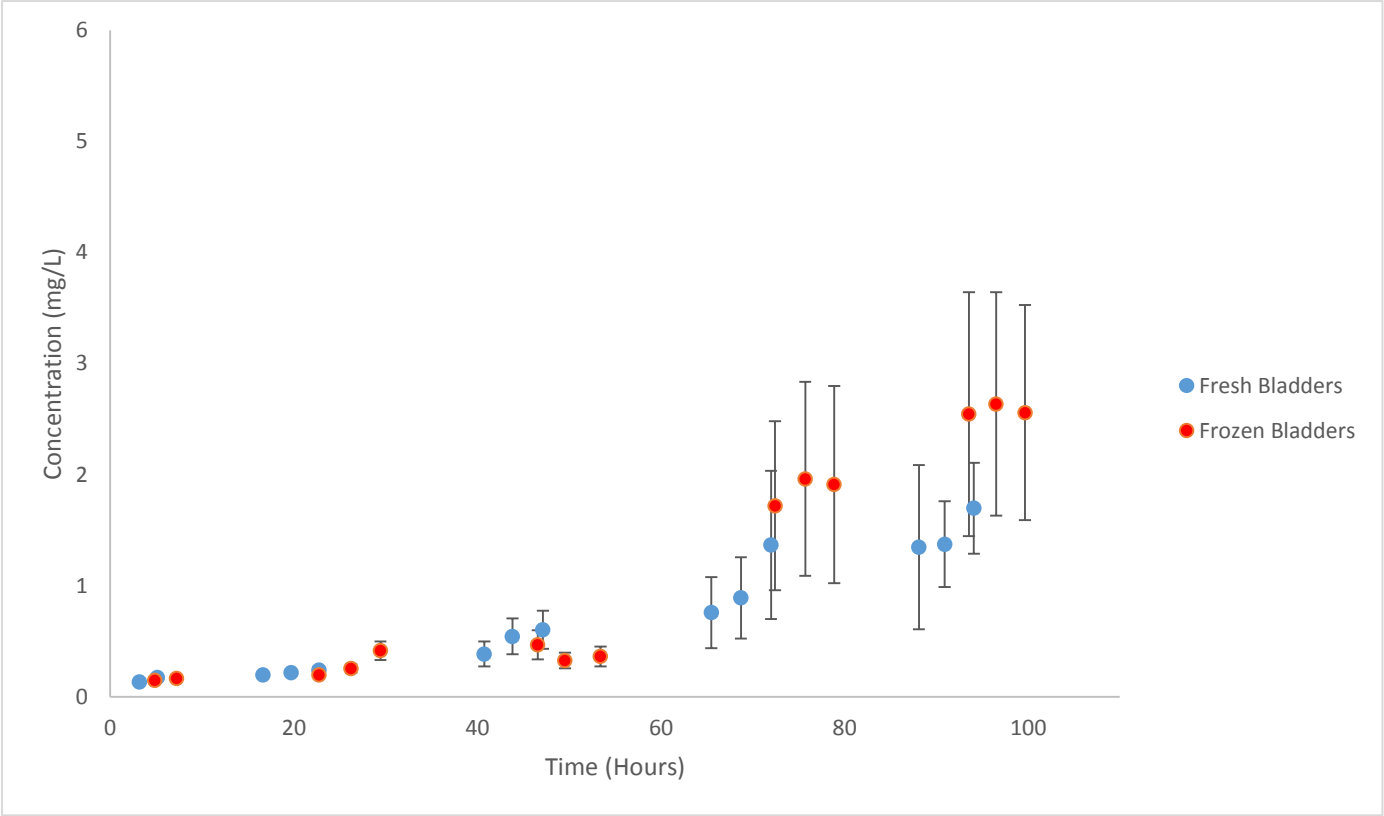

Figure 4.38 Average rhodamine B cumulative concentration at 37°C in PBS pH 7.4; comparing tissue degradation (Fresh tissue and thawed tissue after being frozen for one week),using six inter-bladder sections (n=6) (Error bars are ± S.D.)

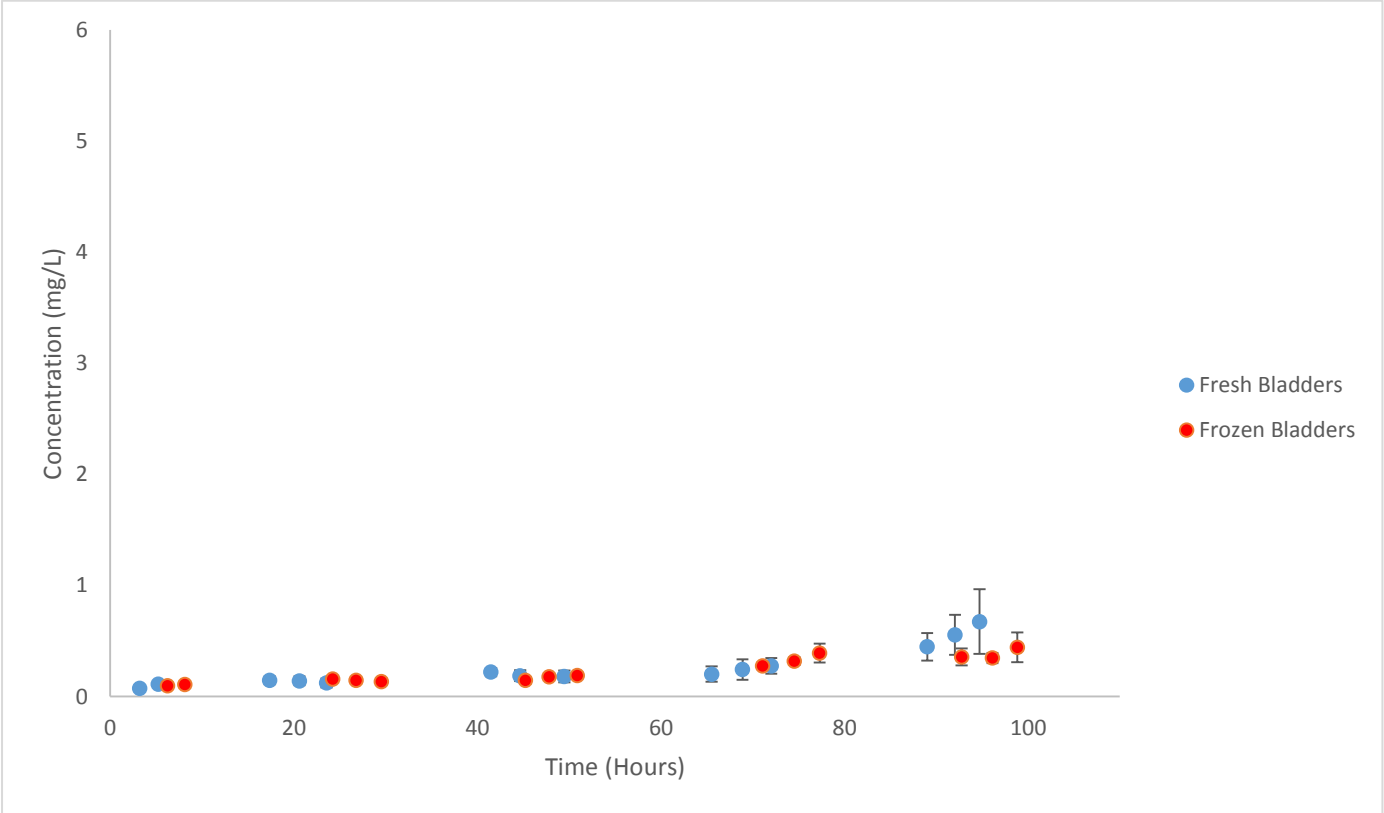

Figure 4.39 Average rhodamine B cumulative concentration at 20°C in PBS pH 7.4; comparing tissue degradation (Fresh tissue and thawed tissue after being frozen for one week), using six inter-bladder sections (n=6) (Error bars are ± S.D.)

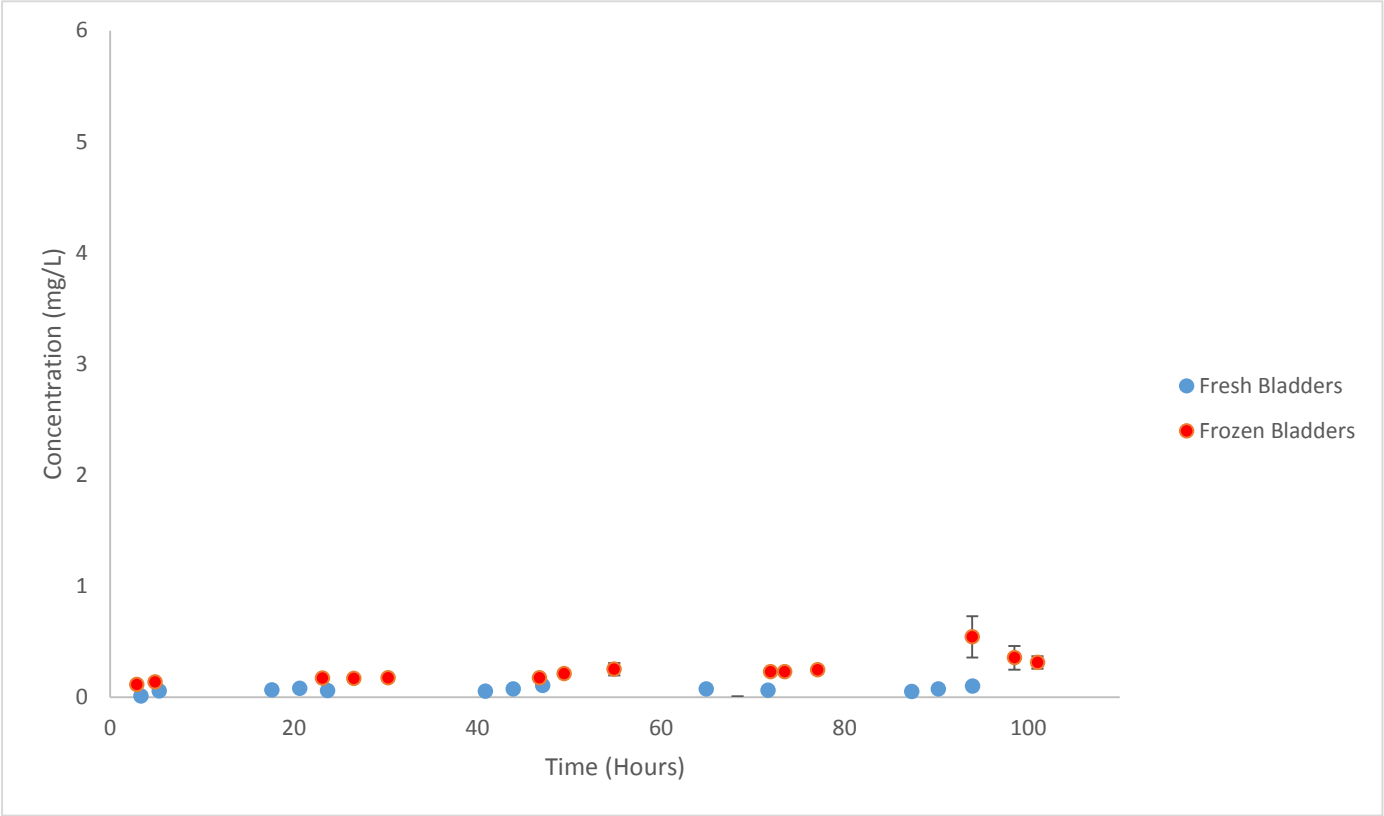

Figure 4.40 Average rhodamine B cumulative concentration at 5°C in PBS pH 7.4; comparing tissue degradation (Fresh tissue and thawed tissue after being frozen for one week), using six inter-bladder sections (n=6) (Error bars are ± S.D.)

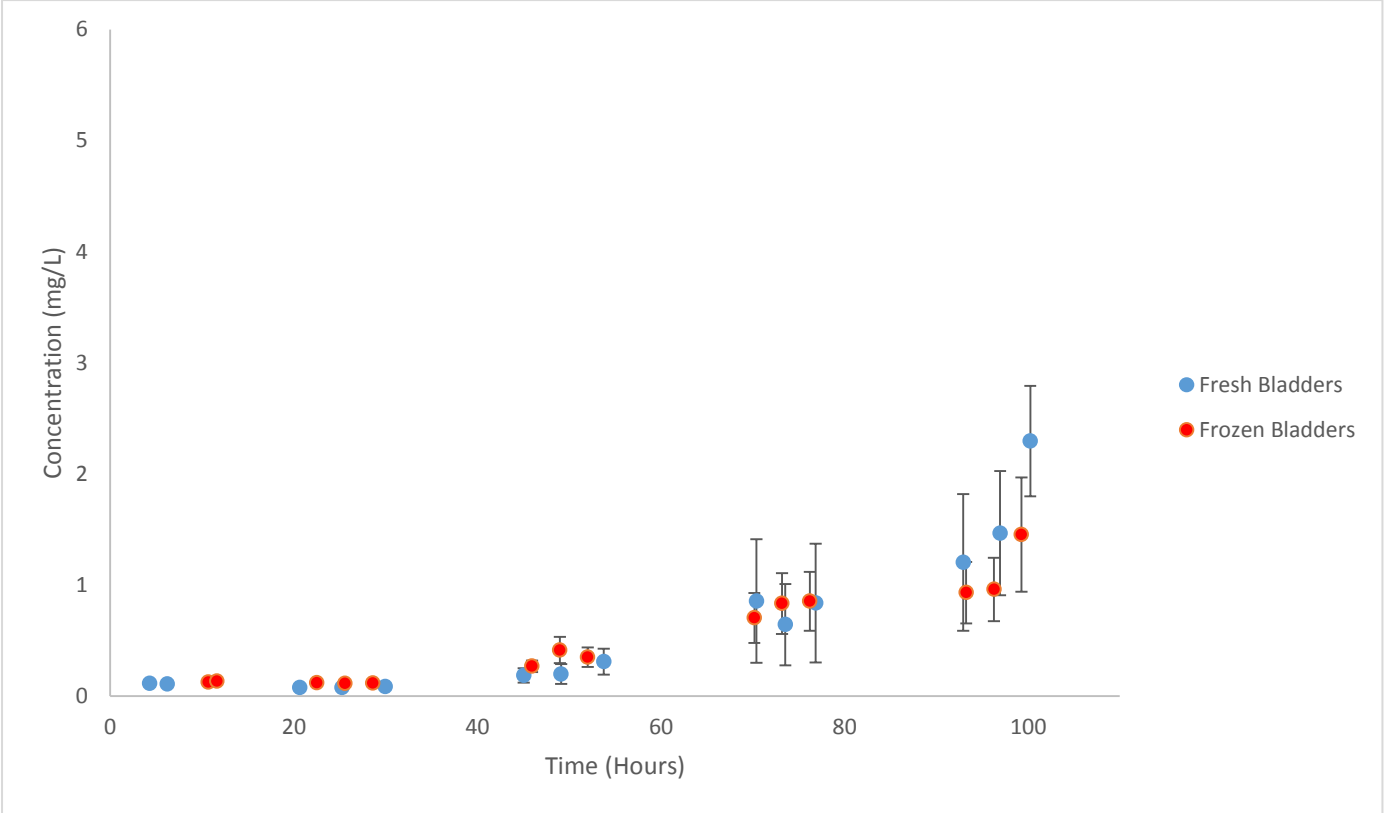

Figure 4.41 Average rhodamine B cumulative concentration at 37°C (pH 5); comparing tissue degradation (Fresh tissue and thawed tissue after being frozen for one week), using six inter-bladder sections (n=6) (Error bars are ± S.D.)

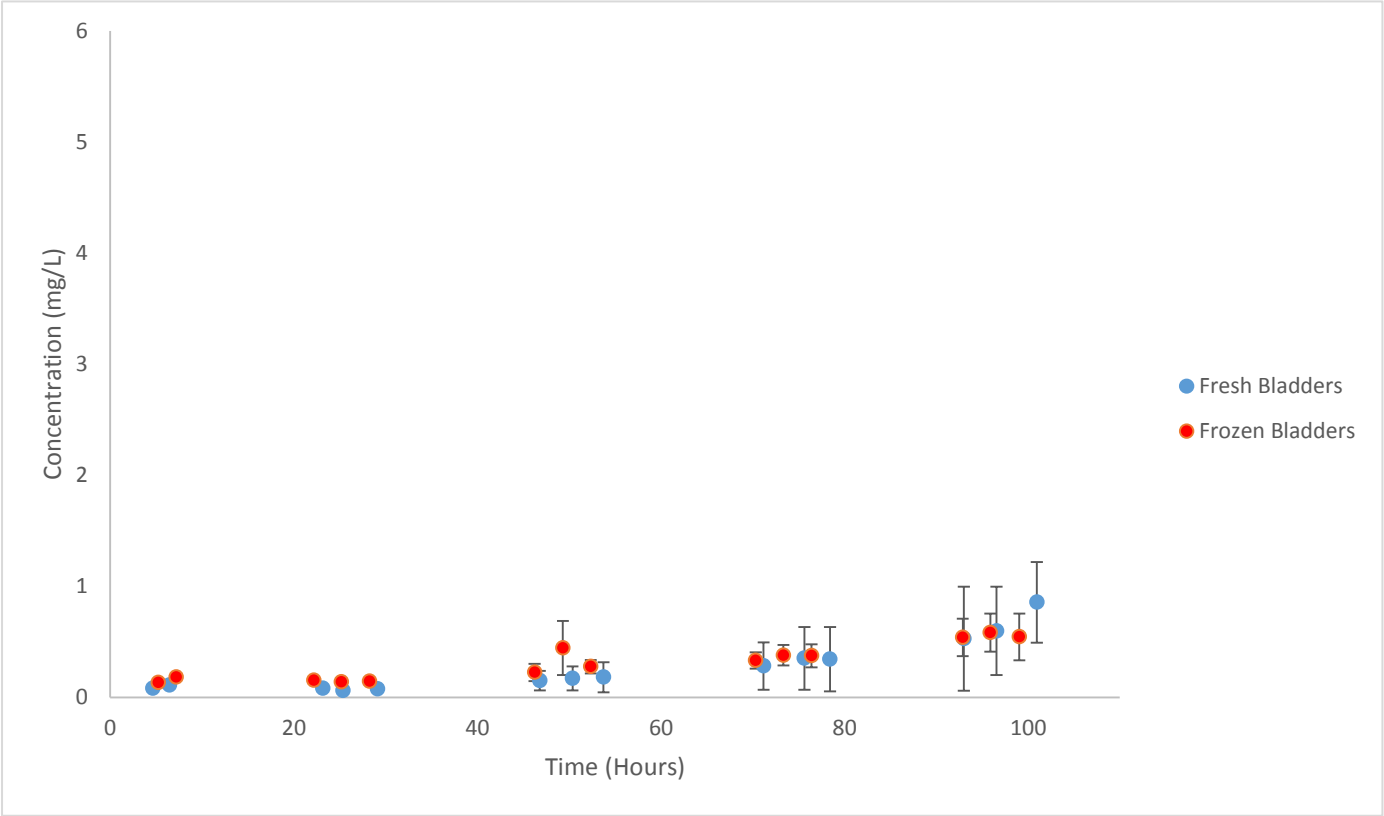

Figure 4.42 Average rhodamine B cumulative concentration at 20°C (pH 5); comparing tissue degradation (Fresh tissue and thawed tissue after being frozen for one week), using six inter-bladder sections (n=6) (Error bars are ± S.D.)

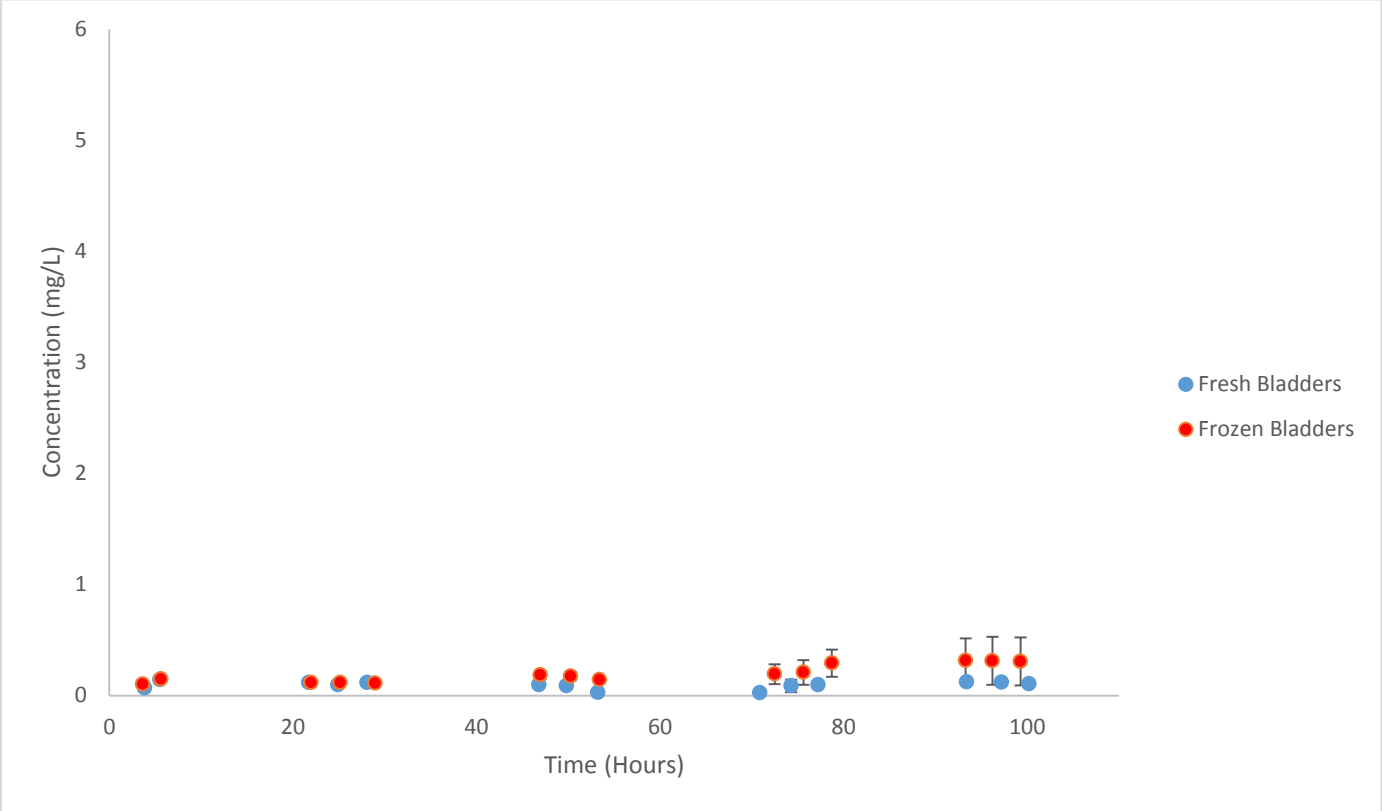

Figure 4.43 Average rhodamine B cumulative concentration at 5°C (pH 5); comparing tissue degradation (Fresh tissue and thawed tissue after being frozen for one week), using six inter-bladder sections (n=6) (Error bars are ± S.D.)

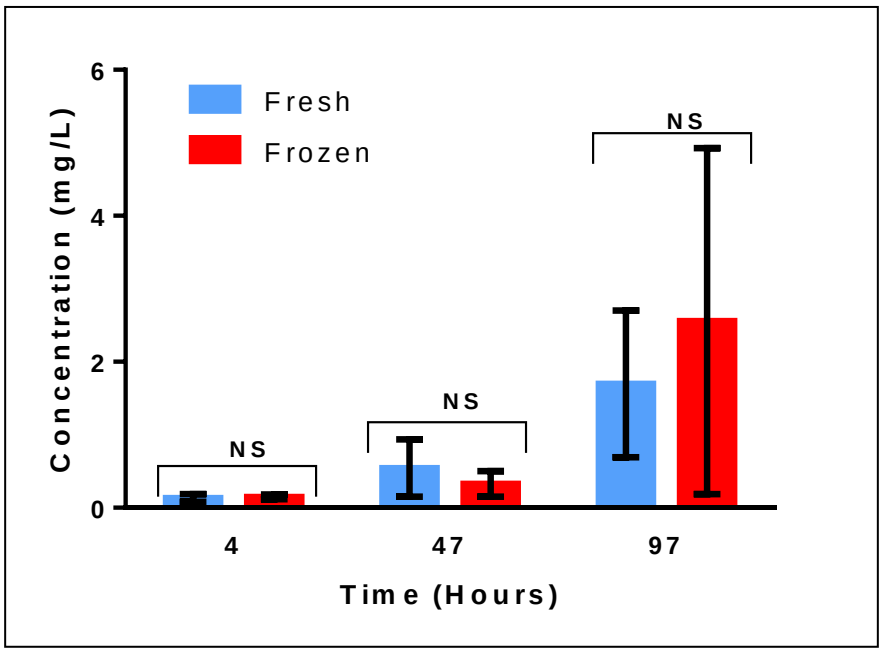

Figure 4.44 Two-Way ANOVA results of rhodamine B concentration in relation to tissue degradation (Fresh tissue and thawed tissue after being frozen for one week) in pH 7.4 at 37°C, using 100 mg/L rhodamine B over approximately 100 hrs, using six inter-bladder sections (Error bars are ± S.D.)

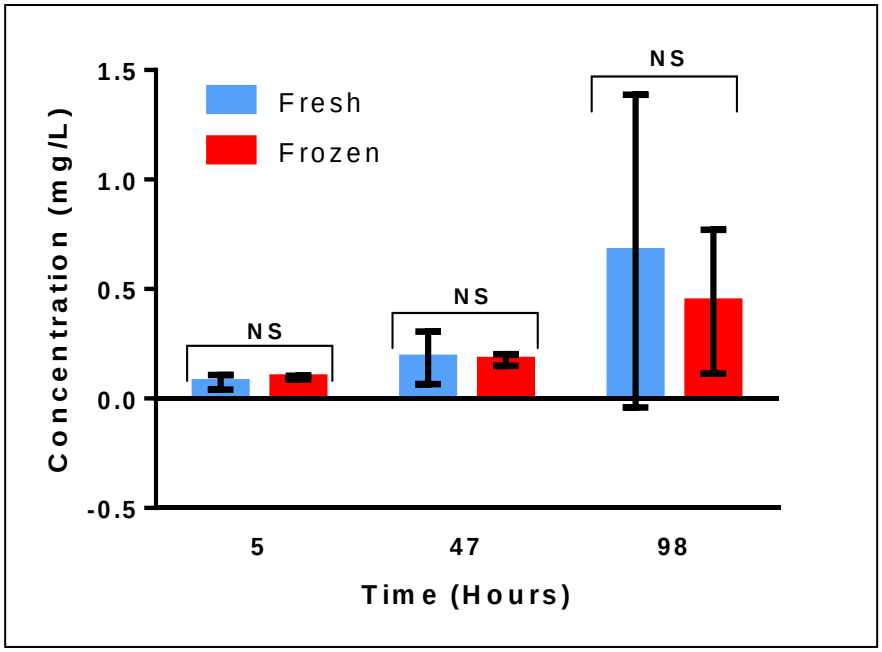

Figure 4.45 Two-Way ANOVA results of rhodamine B concentration in relation to tissue degradation (Fresh tissue and thawed tissue after being frozen for one week) in pH 7.4 at 20°C, using 100 mg/L rhodamine B over approximately 100 hrs, using six inter-bladder sections (Error bars are ± S.D.)

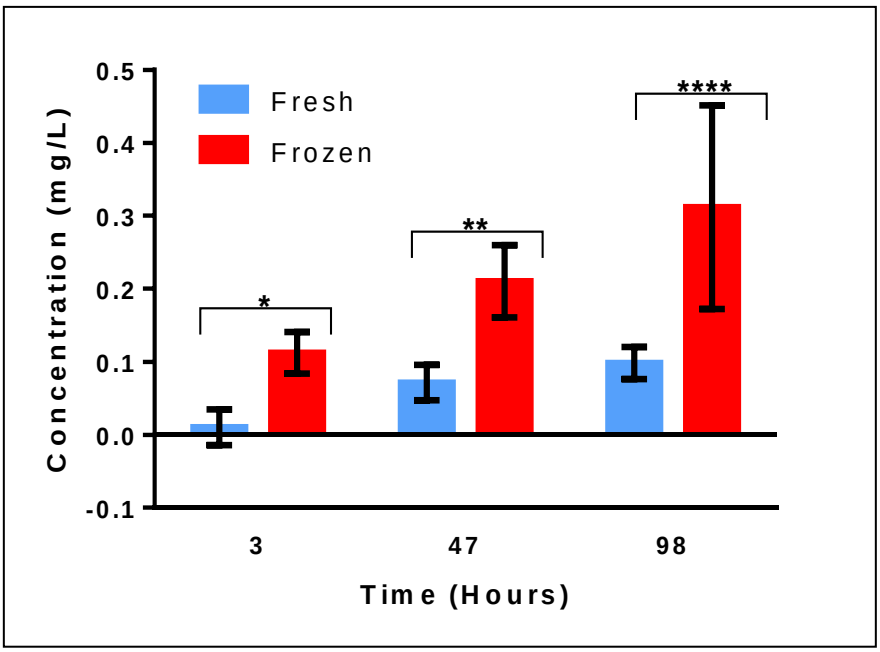

Figure 4.46 Two-Way ANOVA results of rhodamine B concentration in relation to tissue degradation (Fresh tissue and thawed tissue after being frozen for one week) in pH 7.4 at 5°C, using 100 mg/L rhodamine B over approximately 100 hrs, using six inter-bladder sections (Error bars are ± S.D.)

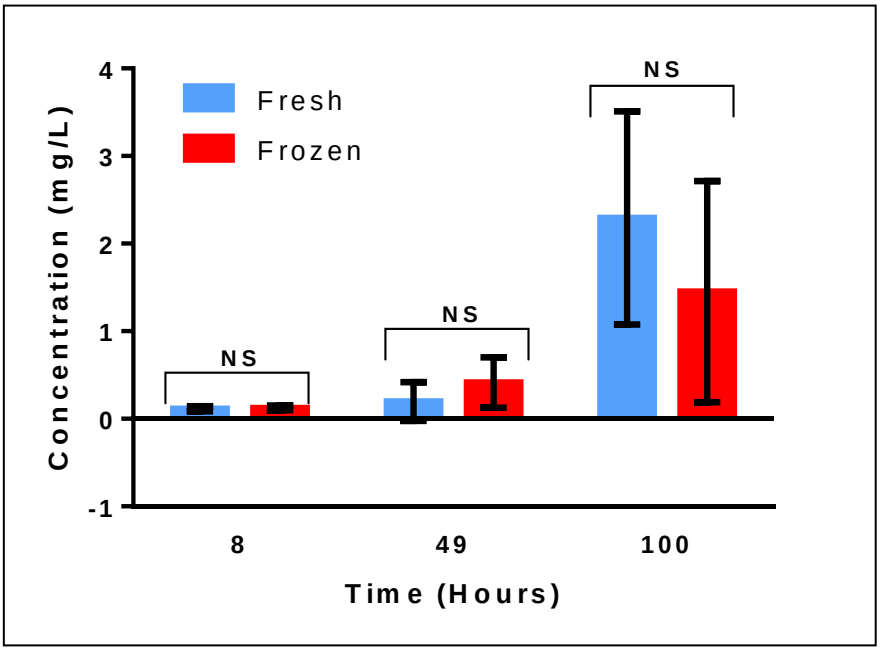

Figure 4.47 Two-Way ANOVA results of rhodamine B concentration in relation to tissue degradation (Fresh tissue and thawed tissue after being frozen for one week) (pH 5) at 37°C, using 100 mg/L rhodamine B over approximately 100 hrs, using six inter-bladder sections (Error bars are ± S.D.)

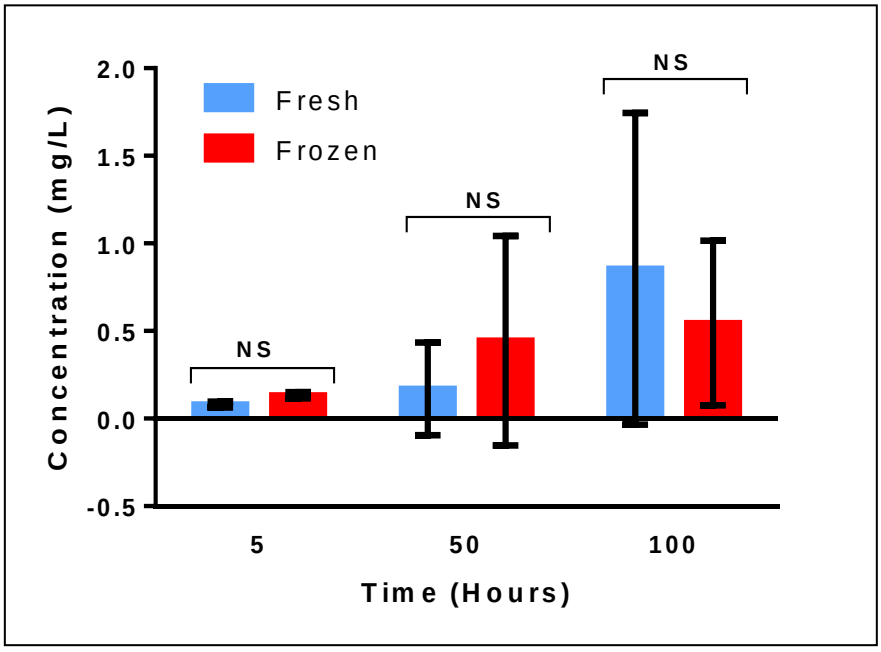

Figure 4.48 Two-Way ANOVA results of rhodamine B concentration in relation to tissue degradation (Fresh tissue and thawed tissue after being frozen for one week) (pH 5) at 20°C, using 100 mg/L rhodamine B over approximately 100 hrs, using six inter-bladder sections (Error bars are ± S.D.)

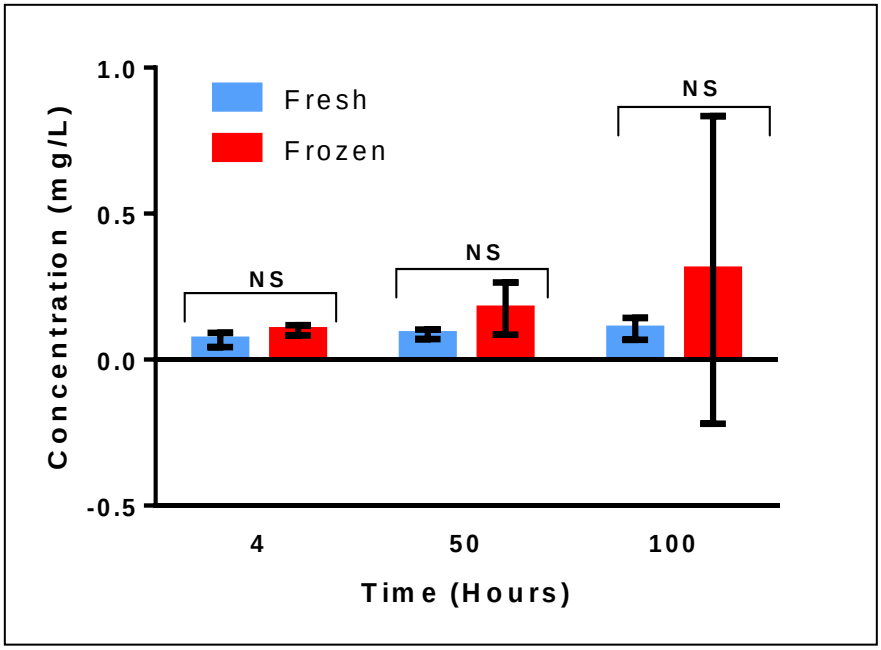

Figure 4.49 Two-Way ANOVA results of rhodamine B concentration in relation to tissue degradation (Fresh tissue and thawed tissue after being frozen for one week) (pH 5) at °C, using 100 mg/L rhodamine B over approximately 100 hrs, using six inter-bladder sections (Error bars are ± S.D.)

4.3.4 Comparison of initial and final Franz cell drug concentrations

The bladder diffusion studies all started with the same concentration of rhodamine B and the two antidepressant drugs (amitriptyline and nortriptyline) at 100 mg/L in the donor chamber. At the end of the experiments, the donor chamber was diluted and analysed to determine the concentration in the donor chamber after the experiment. This was carried out to determine if there was a decrease in the donor chamber and if this loss would equal the concentration found in the acceptor chamber. If there is a difference in concentration that cannot be explained by the concentration in the acceptor chamber this could be due to accumulation of the drug into the tissue (Hilberg *et al.*, 1992) and also possibly from drug degradation. The magnitude of absorption could alter by the ionisation of the drug in different pH solutions.

4.3.4.1 Rhodamine B

There is a general trend of rhodamine B concentration with higher concentrations found in the donor chamber at the end of the experiments when the experimental temperature was lower. This shows less rhodamine B diffused from the donor chamber at the lower temperatures, with 55.17 mg/L in the donor chamber for the fresh inter-bladders at 5°C against 8.68 mg/L at 37°C (See Table 4.2). There are two sets of results that do not support this, showing no trend in donor chamber concentration, which were fresh intra-bladder at pH 7.4 and degraded bladders at pH 5. There were similar concentrations of rhodamine B at 37°C and 5°C (pH 7.4) and higher amount of rhodamine B at 20°C. Showing that at 20°C there was less diffusion from within the same fresh bladder at pH 7.4 and degraded bladders at pH 5. The same trend was shown for the final receptor chamber sample. As a result, there is a large difference between the combined concentrations of rhodamine B against the original donor chamber concentration (100mg/L). This could show that rhodamine B has a high affinity for the bladder tissue and most of the drug was retained in the bladder tissue during diffusion or degradation of the compound over the 100 hrs (See Table 4.2). In addition, the similar results for the intra-bladders show that temperature and pH have less of an effect within the same bladder than between different bladder sections. As a result, the highest temperature (37°C) shows more diffusion from the donor chamber as there is a large decrease in rhodamine B concentration in the final donor chamber. Therefore, more rhodamine B has diffused from the donor chamber into the bladder tissue and then either accumulated the higher amount of rhodamine B in the tissue or degraded. This results in a large difference in rhodamine B concentration when compared to the original 100 mg/L donor chamber concentration.

Table 4.2 Final donor and receptor concentrations for rhodamine B for all bladder studies

	Concentration (mg/L)			
Experiment	**Final Donor**	**Final Receptor**	**Combined**	**Difference from 100mg/L**
Fresh Inter-Bladder, pH 7.4				
37°C	8.68	1.70	10.38	89.62
20°C	37.11	0.67	37.78	62.22
5°C	55.17	0.10	55.27	44.73
Fresh Inter-Bladder, pH 5				
37°C	26.51	2.29	28.80	71.20
20°C	51.97	0.86	52.82	47.18
5°C	56.05	0.11	56.15	43.85
Fresh Intra-Bladder, pH 7.4				
37°C	52.93	1.71	54.64	45.36
20°C	85.80	2.73	88.53	11.47
5°C	54.22	0.01	54.23	45.77
Fresh Intra-Bladder, pH 5				
37°C	29.05	3.38	32.43	67.57
20°C	67.25	0.13	67.38	32.62
5°C	70.81	0.08	70.89	29.11
Degraded Inter-Bladder, pH 7.4				
37°C	20.41	2.56	22.97	77.03
20°C	46.75	0.44	47.19	52.81
5°C	52.69	0.31	53.00	47.00
Degraded Inter-Bladder, pH 5				
37°C	41.06	1.45	42.51	57.49
20°C	48.50	0.55	49.05	50.95
5°C	47.24	0.31	47.55	52.45

4.3.4.2 amitriptyline and nortriptyline

There were opposite results for amitriptyline and nortriptyline with increasing concentrations linked to decreasing temperature for amitriptyline, which was consistent with rhodamine B (See Table 4.3). However, nortriptyline showed decreasing concentrations with lower temperatures, demonstrating more diffusion as the temperature decreased (See Table 4.4). However, the concentration difference between original donor chamber and combined final concentrations were very large, with an average final concentration for pH 7.4 of 28.09 mg/L and 23.23 mg/L for pH 5, showing that both drugs possibly also had high affinity for the bladder tissue. As an example, the fresh inter-bladder at pH 7.4 the average difference was 79.91 mg/L and 76.77 mg/L for pH 5, showing a large portion of the drug could have a high affinity for the biological tissue.

Table 4.3 Final donor and receptor concentrations for amitriptyline for all bladder studies

Amitriptyline	**Concentration (mg/L)**			
Experiment	**Final Donor**	**Final Receptor**	**Combined**	**Difference from 100mg/L**
Fresh Inter-Bladder, pH 7.4				
37°C	19.04	1.65	20.69	79.31
20°C	26.10	0.61	26.72	73.28
5°C	36.37	0.49	36.86	63.14
Fresh Inter-Bladder, pH 5				
37°C	39.35	0	39.35	60.65
20°C	19.04	0	19.04	80.97
5°C	11.30	0	11.30	88.70

Table 4.4 Final donor and receptor concentrations for nortriptyline for all bladder studies

Nortriptyline	**Concentration (mg/L)**			
Experiment	**Final Donor**	**Final Receptor**	**Combined**	**Difference from 100mg/L**
Fresh Inter-Bladder, pH 7.4				
37°C	46.54	5.13	51.67	48.33
20°C	26.37	0	26.37	73.64
5°C	41.29	0.28	41.57	58.43
Fresh Inter-Bladder, pH 5				
37°C	39.79	1.69	41.48	58.52
20°C	17.58	0	17.58	82.43
5°C	8.42	0	8.42	91.58

4.3.5 Comparison of bladder tissue thickness recorded from Franz cell diffusion studies

The bladder is a storage organ and can store a variable volume of solution, for this to occur the bladder expands to hold the extra volume. As a result, the bladder wall stretches and this reduces the thickness of the bladder membrane, human bladder wall thicknesses have been measured with 1.9 ± 0.4 mm for women and 2.1 ± 0.5 mm for men (Kanyilmaz, Calis, Cinar, 2013). Distention has been said to reduce the diffusion of compounds as a result, the bladder thicknesses that would be affected with a distended bladder was investigated using this experiment (Borzelleca and Lowenthal, 1967). Six bladders were used for the inter-bladder experiments with one section removed from the same area within the bladder to be analysed on the Franz cells (See Section 2.2.1.1.1). To determine the variation in thickness within the same bladder, one bladder was sectioned into four and all four sections used in the diffusion experiments (See Section 2.2.1.2). In addition, degradation was a factor that was investigated in the experiments as a result six bladders were frozen for one week prior to being analysed using rhodamine B (See Section 2.2.1.1.2). The thickness of all the bladder sections in these experiments was measured using digital callipers. This was to determine if there was significant difference between bladder thicknesses, which could result as this becoming a factor that could affect the rate of diffusion of compounds over the post-mortem period.

4.3.5.1 Rhodamine B, amitriptyline, and nortriptyline inter-bladder diffusion studies

The bladders are in numerous stages of storing urine with 50% of cases voiding the bladder at the time of death (Negrusz, 2013). After excising the bladder and removal of the urine, the bladder does not contract to the original size when there is no urine present. As a result, the bladder membrane can be a range of thicknesses; this comparison is used to determine if the range of thicknesses are significant and therefore could be classed as a factor that could affect the rate of drug diffusion.

There was a range of different bladder thicknesses with the mean thickness from 108 measurements at 4.74 mm ± 1.35 mm. The histogram (See Figure 4.50) shows the data to be close to symmetric, this is also confirmed as the mean, and median values are similar with values at 4.74 mm and 4.8 mm respectively. The standard deviation is approximately 30% of the mean showing there is variation in the bladder thickness. The range of bladder thicknesses is 7.53 mm however, the 75% percentile shows most of the data are below 5.49 mm. With 95% confidence the bladder thickness range was between 4.48 and 5.0 mm.

As can be seen from figure 4.51 there was no significant difference (p 0.42) between the thickness of fresh or degraded bladders (mean bladder thicknesses, fresh bladders: 4.8 ± 1.3 mm and frozen bladders: 5.6 ± 1.1 mm). As a result it is unlikely that the differences in bladder thickness are the likely to be a significant cause of the diffusion difference between the fresh or degraded bladders.

4.3.5.2 Rhodamine B intra-bladder diffusion studies

The sections within the same bladder were taken for each experiment and as a result, the thicknesses recorded for the top of the bladder were compared to the thickness recorded for the bottom of the bladder to determine if there is significant difference between different areas within the same bladder. The results had a p value of 0.23 and therefore showed no significant difference (See Figure 4.52) between the top and bottom bladder sections (mean bladder thicknesses, top sections: 4.2 ± 1.0 mm and bottom sections: 4.6 ± 1.4 mm).

This shows that the bladder thicknesses within the same bladder could not be classed as a factor that could affect drug diffusion.

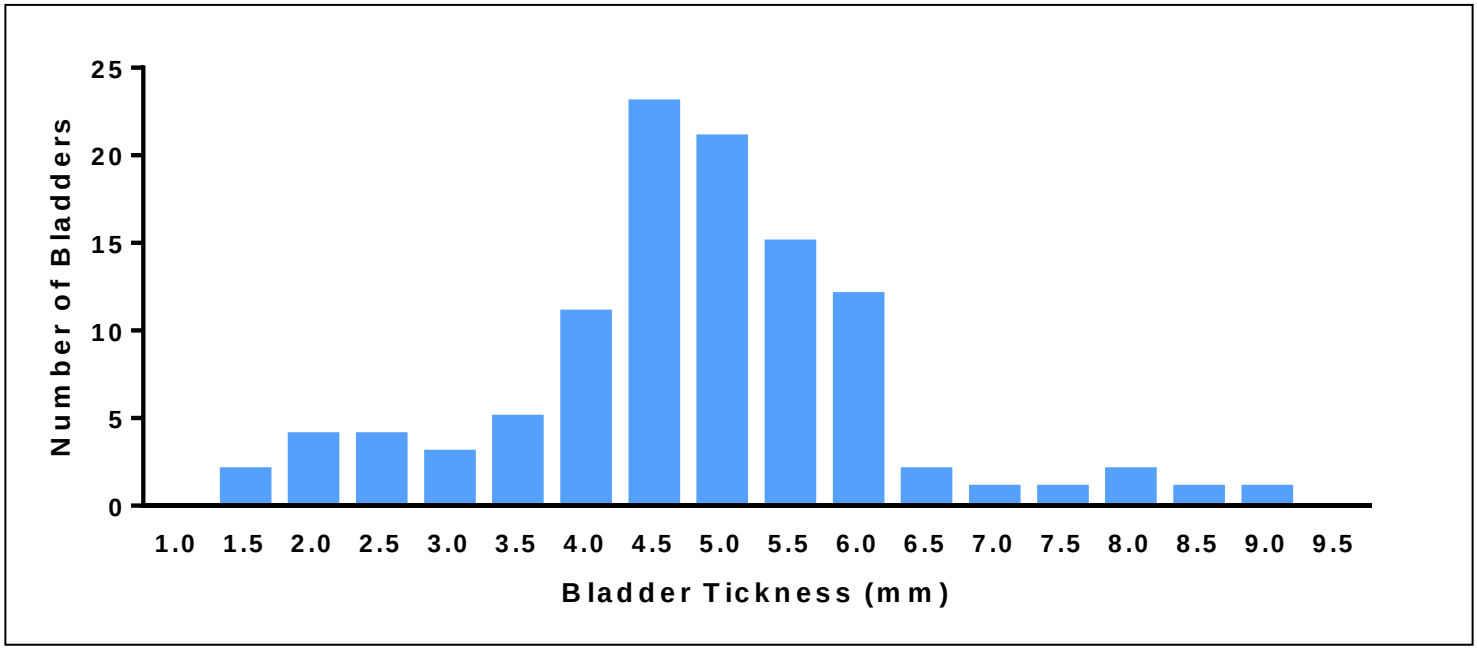

Figure 4.50 Histogram of the range of bladder thicknesses from the inter-bladder diffusion experiments (n=108)

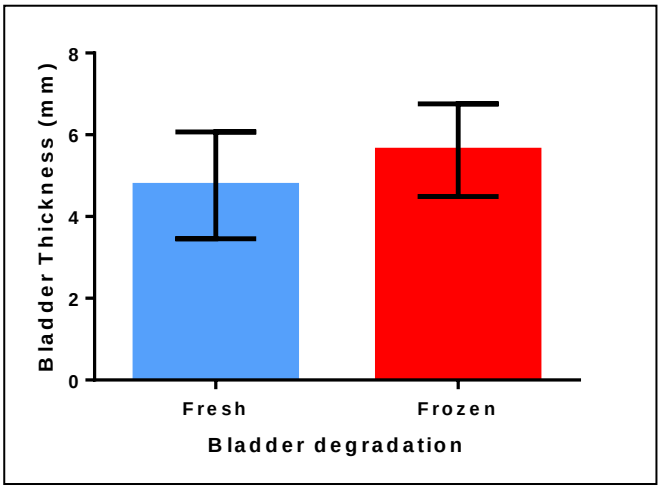

Figure 4.51 Bladder thickness comparison between the fresh and degraded inter-bladder sections (Fresh bladders =36 and frozen bladders = 31) (Error bars are ± S.D.)

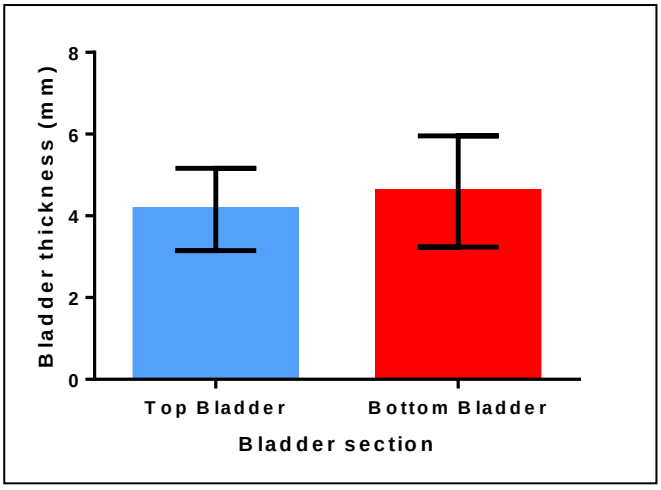

Figure 4.52 Bladder thickness comparison between the two top sections of the bladder and the two bottom sections of the bladder (Top and bottom bladders = 18) (Intra-bladder diffusion studies) (Error bars are ± S.D.)

4.3.6 Comparison of drug permeability using calculated values from the bladder diffusion studies

In order to obtain a better idea of the diffusion of the drugs (rhodamine B, amitriptyline, and nortriptyline) the flux (describes the mass movement of molecules through a membrane) of each drug across the bladder was calculated. The flux value is needed to calculate the permeability, which can be calculated in two different ways dependant on the amount of drug diffusing through the membrane (See Section 2.3.1). The permeability coefficient of a compound in a state where there is < 10% movement of the analyte into the acceptor chamber from the donor chamber is known as steady-state, which has a constant concentration gradient resulting in constant flux. This directly relates the concentration gradient and the flux (See Section 2.3.1). Drugs can be compared using permeability when experiments have been carried out at numerous concentration gradients. However, if more than 10% of the original donor chamber concentration is transferred this results in non-steady state conditions. The flux changes to first order when under non-steady state conditions. Non-steady state conditions can occur with lipophilic drugs or when the experiment is undertaken for over a long period. The equations become more complicated as the flux is not constant and cannot be directly equated to the permeability. Other parameters are needed including donor and receptor concentrations as the donor chamber concentration will significantly decrease. The permeability is being determined from experimental values, which can be affected by a number of parameters and can experience bias. Therefore, instead of labelling permeability as P, the label is changed to $P_{effective}$ as this is highlighting the permeability value is being experimentally obtained instead of using models (Brodin, Steffansen and Nielsen, 2010).

4.3.6.1 Rhodamine B

The results were taken from the bladder diffusion studies to determine the permeability of rhodamine B at all the parameters investigated in the bladder studies. The permeability increases by a factor of ten at the highest temperature (37°C), with $1.8x10^{-4}$ cm/s (Fresh inter-bladder pH 7.4) at 37°C in comparison with $6.8x10^{-5}$ cm/s at 20°C and $1.4x10^{-5}$ cm/s at 5°C at pH 7.4 as an example. In all three sets of experiments, this shows that high temperatures do affect the permeability of rhodamine B (See Table 4.5). The fresh inter-bladder studies have consistent results at both pH's showing a decrease in permeability as the temperature decreases. The similar values show there is no preferential diffusion dependant on pH. There is no clear trend in the change in permeability for the intra-bladder studies however; the highest permeability was calculated for the highest temperature (37°C) consistent with the inter-bladder studies (See Table 4.5). The comparison of permeability values between pH shows there is more diffusion at pH 5 than at pH 7.4. The degraded tissue experiments have similar results in PBS showing the highest permeability at 37°C and no difference in permeability between 20 and 5°C. The pH 5 results show a trend in decreasing permeability as the temperature decreases (See Table 4.5).

Table 4.5 Permeability values of rhodamine B for all experimental parameters in the bladder studies

Experiment	Number of samples used to calculate permeability	Permeability Value (cm/s)
Fresh, Inter-Bladder:		
pH 7.4, 37°C	12	$1.8x10^{-4}$
pH 7.4, 20°C	12	$6.8x10^{-5}$
pH 7.4, 5°C	9	$1.4x10^{-5}$
pH 5, 37°C	6	$1.8x10^{-4}$
pH 5, 20°C	12	$5.7x10^{-5}$
pH 5, 5°C	12	$2.6x10^{-5}$
Fresh, Intra-Bladder:		
pH 7.4, 37°C	7	$3.0x10^{-4}$
pH 7.4, 20°C	9	$3.5x10^{-4}$
pH 7.4, 5°C	11	$1.1x10^{-5}$
pH 5, 37°C	6	$5.8x10^{-4}$
pH 5, 20°C	12	$3.0x10^{-5}$
pH 5, 5°C	12	$3.1x10^{-5}$
Degraded Inter-Bladder:		
pH 7.4, 37°C	6	$3.6x10^{-4}$
pH 7.4, 20°C	12	$5.8x10^{-5}$
pH 7.4, 5°C	12	$5.9x10^{-5}$
pH 5, 37°C	8	$1.4x10^{-4}$
pH 5, 20°C	12	$7.7x10^{-5}$
pH 5, 5°C	12	$3.6x10^{-5}$

4.3.6.2 amitriptyline and nortriptyline

As can been seen in table 4.6 and 4.7 the permeability values for amitriptyline and nortriptyline are of the same magnitude independent of the pH and temperature. The permeability in pH 7.4 is lower at 37°C and similar at the two lower temperatures (20°C and 5°C). These results could be due to the higher temperature (37°C) being under non-steady state conditions whereas all the lower temperatures are under steady state conditions. There is only one result for pH 5, which shows slightly more permeability than at pH 7.4, resulting in more permeability of amitriptyline at the lower pH. However, nortriptyline at the lower pH 5 shows similar permeability values at the two higher temperatures and a lower value at 5°C, showing the lower temperature reduces the permeability of nortriptyline. In addition, the nortriptyline results are consistent with amitriptyline concerning the pH, as there is more permeability of nortriptyline at the same high temperature (37°C) and the lower pH. This is not expected for either drug as the lower pH would ionise the drugs further than at pH 7.4, which should reduce the capacity of the drugs to diffuse through the tissue membrane.

Table 4.6 Permeability values of amitriptyline for all experimental parameters in the bladder studies

Experiment	Number of samples used to calculate permeability	Permeability Value (cm/s)
Fresh, Inter-Bladder:		
pH 7.4, 37°C	12	1.9×10^{-5} (Non-Steady State)
pH 7.4, 20°C	10	9.7×10^{-5}
pH 7.4, 5°C	9	6.9×10^{-5}
pH 5, 37°C	6	3.4×10^{-5}

Table 4.7 Permeability values of nortriptyline for all experimental parameters in the bladder studies

Experiment	Number of samples used to calculate permeability	Permeability Value (cm/s)
Fresh, Inter-Bladder:		
pH 7.4, 37°C	9	3.0×10^{-5} (Non-Steady State)
pH 7.4, 20°C	8	3.0×10^{-5}
pH 7.4, 5°C	6	1.4×10^{-5}
pH 5, 37°C	8	4.6×10^{-5}

4.3.7 Acceptor chamber pH change over 100 hrs

The pH was recorded at the start and end of the bladder diffusion studies to observe any pH changes, and if these changes were random or followed a trend. This is due to after death the pH within the body changes overtime due, in part, to anaerobic respiration. These pH recording was taken to determine if the same pH changes can be observed *in vitro*.

Table 4.8 Final acceptor chamber pH values

	pH values	
Experiment	**7.4**	**5.0**
Inter-Bladder, Control		
37°C	6.87 ± 0.24	6.95 ± 0.16
20°C	6.99 ± 0.13	5.31 ± 0.02
5°C	7.10 ± 0.08	5.16 ± 0.02
Inter-Bladder, rhodamine B		
37°C	-	6.88 ± 0.40
20°C	6.55 ± 0.07	5.33 ± 0.08
5°C	7.02 ± 0.08	5.20 ± 0.03
Intra-Bladder, rhodamine B		
37°C	6.81 ± 0.14	7.60 ± 0.57
20°C	6.69 ± 0.11	5.28 ± 0.07
5°C	7.15 ± 0.23	5.08 ± 0.01
Inter-Bladder, rhodamine B (Degraded)		
37°C	7.26 ± 0.41	-
20°C	6.69 ± 0.06	5.37 ± 0.05
5°C	7.03 ± 0.02	5.24 ± 0.04
Inter-Bladder, Mixed Drugs		
37°C	6.84 ± 0.24	6.84 ± 0.33
20°C	6.99 ± 0.13	5.19 ± 0.24
5°C	7.10 ± 0.08	6.53 ± 0.05

The table (See Table 4.8) shows the final pH values from the acceptor chamber solutions at both pH (PBS and AA) for the diffusion studies, the values seem to follow a trend that is independent of the solution pH. The PBS solution results show the pH is consistently lower than the original pH 7.4 value at the start of the experiment. This is inconsistent with current knowledge that the pH undergoes limited changes after death; these results could be due to the replacement of the acceptor chamber solution with the blank pH solution that was altered with differing amounts of acid or base to rectify the pH during the experiment. The AA solution results show the pH value consistently increases over the course of the experiment, which coincides with the later stages of decomposition after autolysis, where the putrefaction stage can show increase in pH due to bacteria. The trend that is present is independent of solution pH, different tissue conditions (fresh and degraded), and different drugs present. This trend shows, even with opposite changes in pH, the most change is in the highest temperature experiments and the lowest temperature shows the minimal pH change. As a result, the temperature can be shown to be linked to pH where the higher the temperature results in the

larger alteration of pH in post-mortem porcine bladder tissue, which is probably due to increased decomposition from the increased temperature (Cockle and Bell, 2015). However, there is a limitation to this experiment as there were a number of samples taken from the Franz cells each day and replaced with blank solution. Fresh PBS or AA added to the chambers each day that had been altered by an acid or base at difference concentrations so the same ionic strength of the solution was not added and this could have had an adverse effect on the final pH.

4.3.8 Interference peaks on the HPLC

Overtime, interference peaks were detected in the validation that co-eluted with the antidepressant drugs, specifically amitriptyline, and increased throughout the diffusion study analysis period. This is possibly due to the limited sample preparation of the solutions taken from underneath the bladder tissue. The only sample preparation included filtration of the samples due to restricted sample volume (See Section 2.2.2.3). As a result, no extraction of any tissue in the samples took place. Therefore, the interference peaks could be due to bladder tissue, proteins or other compounds still present in the samples when analysed on the HPLC.

The UV spectra of the two drugs (amitriptyline and nortriptyline) are shown in figure 4.53 - 4.54 for reference. There were a number of contamination peaks that co-eluted with both drugs, mainly amitriptyline shown in figures 4.55 - 4.56. As a result, the peaks could be identified in the library with a poor match and therefore could not be assigned as amitriptyline. Figure 4.57 shows amitriptyline was more prominent in the UV spectrum and therefore detected in the library at an acceptable match (above 950/1000). The match allowed amitriptyline to be assigned however; it could not be quantified due to co-elution as this could overestimate the amount of drug present. There was also split peaks, which occurred with nortriptyline (See Figure 4.58) and this affected the UV spectra (See Figure 4.59). This prevented identification and quantification of the drug in numerous samples resulting in underestimating the detection of both drugs using the sample preparation and HPLC methods. Future work would involve developing a drug extraction method prior to analysing the samples on the HPLC to achieve cleaner samples.

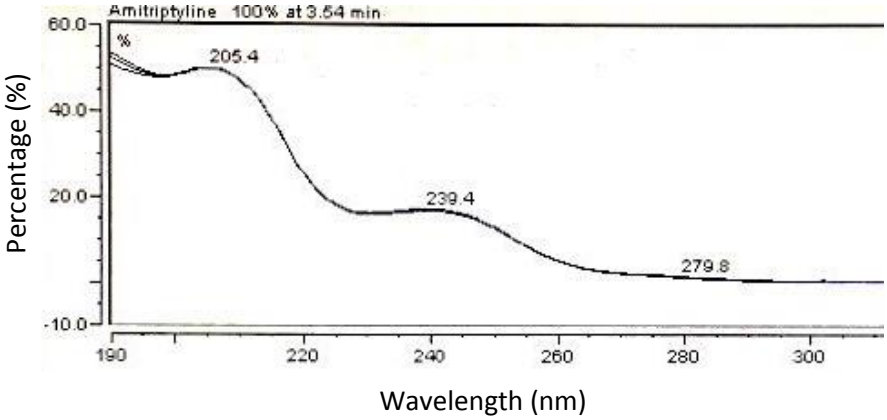

Figure 4.53 UV spectrum of amitriptyline from the Chromeleon library

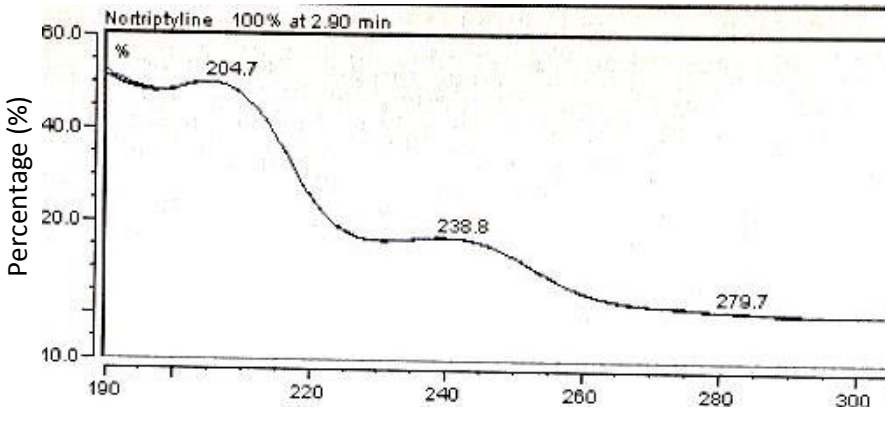

Figure 4.54 UV spectrum of nortriptyline from the Chromeleon library

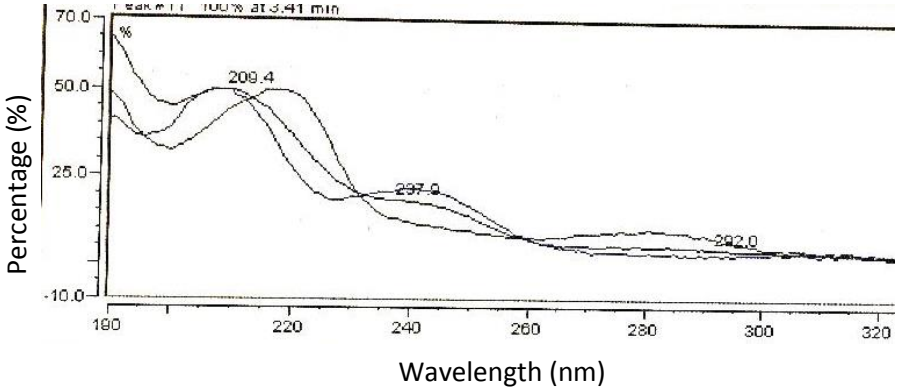

Figure 4.55 UV spectrum of amitriptyline and co-eluting compounds from the bladder section diffusion studies at 37°C (pH 7.4), 47 hrs post-mortem

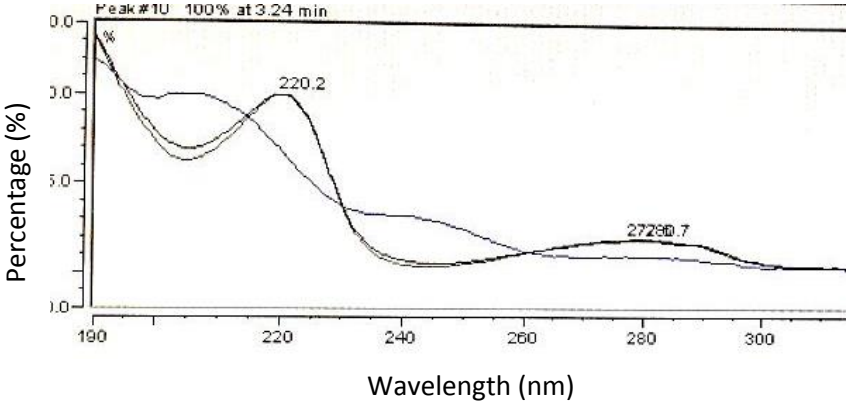

Figure 4.56 UV spectrum of amitriptyline and co-eluting compounds from the bladder section diffusion studies at 37°C (pH 7.4), 50 hrs post-mortem

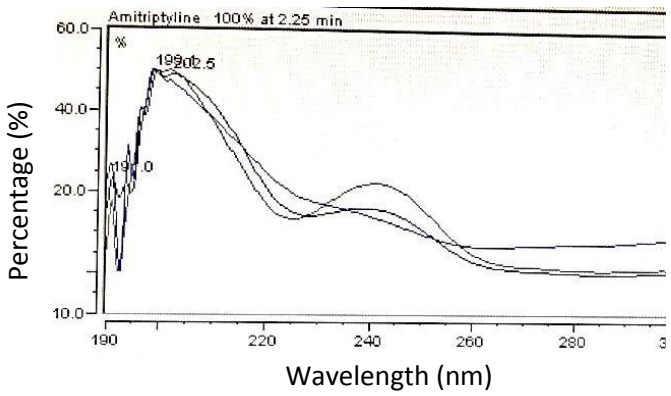

Figure 4.57 UV spectrum of amitriptyline and co-eluting compounds from the bladder section diffusion studies at 20°C (pH 5), 70 hrs post-mortem

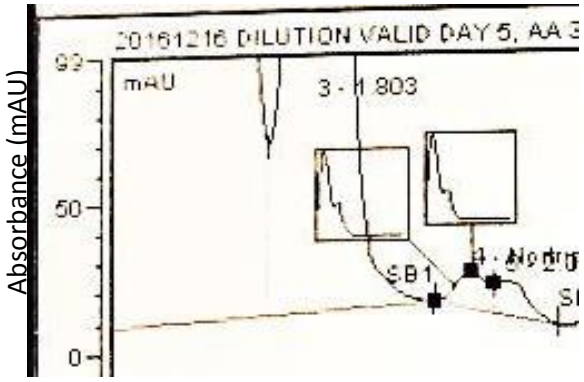

Figure 4.58 Chromatogram showing split peak of nortriptyline from the bladder section diffusion studies at 37°C (pH 5), 49 hrs post-mortem

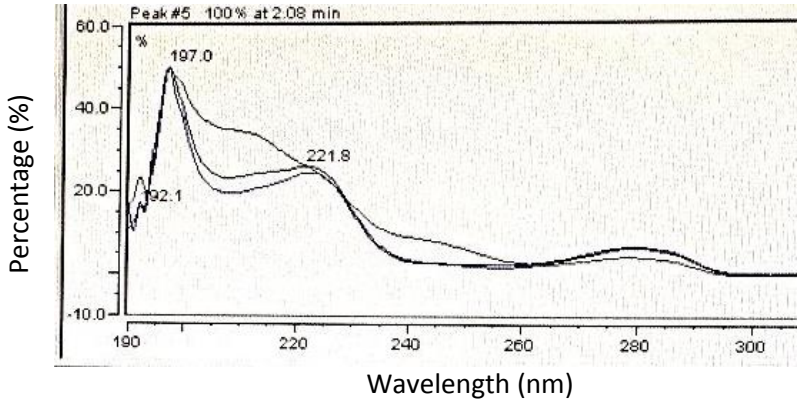

Figure 4.59 UV spectrum of the split peak of nortriptyline and the contaminating compound from the bladder section diffusion studies at 20°C (pH 5), 70 hrs post-mortem

4.4 Discussion

The aim of this chapter was to investigate a number of possible factors that could affect the diffusion of rhodamine B, amitriptyline, and nortriptyline through sections of bladder tissue in order to determine if post-mortem redistribution from the bladder is likely to be a significant problem in forensic toxicology interpretation. These factors included temperature, drug concentration, pH, tissue degradation, and bladder thickness. The results showed that temperature was the only parameter that affected the concentration of rhodamine B that diffused through the bladder tissue. The temperature and pH seemed to have an effect on the concentration of the two antidepressant drugs. The other parameter, involving freezing and thawing the tissue, was not deemed a factor that could affect the concentration of drugs diffusing through the bladder tissue. However, the effect of temperature was limited to a significant increase in diffusion when the bladders were at physiological temperature. However, the body would not be at this temperature for a long period after death due to body cooling, which would occur within the first 18 hrs after death (Fisher, 2003). The significant differences occurred at the later stages in the experiment, approximately 94 ± 0.1 hrs for pH 7.4 ($p < 0.001$) and 100 ± 0.06 hrs for pH 5 ($p > 0.0001$), with higher rhodamine B concentrations from physiological temperature (37°C) in comparison with the two lower temperatures (20°C and 5°C). No significant differences were found between the room temperature (20°C) that the body would equilibrate after death and the mortuary fridge temperature (5°C) where the body would be stored before autopsy. Therefore, after the initial stages of decomposition and the body had cooled, the temperature does not affect the amount of rhodamine B concentration diffusing through the bladder tissue. The peak concentrations achieved at the respective temperatures show that the lower temperatures result in lower rhodamine B concentrations. The fresh inter-bladder results at pH 7.4 had resulting peak concentrations of 1.70 ± 1.0 mg/L at 37°C, 0.67 ± 0.71 mg/L at 20°C and at 5°C the amount of rhodamine B was below the limit of detection of the method. The concentrations at pH 5 was 2.29 ± 1.22 mg/L at 37°C, 0.85 ± 0.89 mg/L at 20°C and the amount of rhodamine B was < the limit of detection for the method at 5°C.

Similar trends were shown for the degraded tissues in relation to temperature, the intra-bladder results have less of a clear trend with a higher peak concentration at 20°C (2.73 ± 2.31 mg/L) than 37°C (1.86 ± 1.75 mg/L). However, in all cases the large standard deviations show there is a wide range of overlap for the rhodamine B cumulative concentrations. On the other hand, there was a general increase of rhodamine B cumulative concentration overtime. The Skopp paper (Skopp *et al.*, 1997) shows there is a longer lag time in the diffusion of rhodamine B through vein tissue at lower temperatures, which is consistent with the results from this research. Opposing research shows the distribution of rhodamine B in liver slices was not affected by temperature, with similar patterns using 37°C or 4°C however, the thickness of the liver slices was much thinner at 0.25 mm (Olinga *et al.*, 2001). Overall, in relation to temperature, the cooler temperatures reduce the cumulative concentration of the three drugs over five day's post-mortem. If an individual was found outside, which could result in cooler conditions between 20°C and 5°C, this lower temperature would reduce diffusion from the bladder.

The pH of the urine can vary with a range of PM urine pH between pH 4.6 – 8.5 (Cook, Strauss and Caplan, 2007), as a result this would be expected to alter the ionisation of drugs, affecting the amount of drugs that

are able to diffuse through the bladder membrane. However, there was no significant difference between the diffusion of rhodamine B, comparing the pH at any temperature for both the fresh and degraded bladder tissue sections, which is consistent with the similar ionisation state of rhodamine B at 99.9% in pH 7.4 and 86% in pH 5. This shows that diffusion is independent of pH, therefore as the body decomposes, and reduces in pH from physiological (pH 7.4) to the more acidic pH (pH 5), the rate of drug diffusion is not affected. The post-mortem pH range stated previously, pH 4.6 – 8.5 (Cook, Strauss and Caplan, 2007), suggests this would be possible in the bladder. Rhodamine B has a negative and positive charge in aqueous solutions however, it is less protonated at higher pH (pH 7.4) and therefore this weakens hydrogen bonds and the hydrophobic forces involved in binding the compound. This results in a higher release rate and these bonds strengthen in acidic environments (pH 5) resulting in a slower drug release rate (Zhang *et al.*, 2011). This could explain the differences in the cumulative concentrations of rhodamine B diffusing through the bladder tissue at pH 7.4 compared with pH 5 with higher concentrations at the higher pH.

The results for amitriptyline and nortriptyline are inconclusive as there were results for all three temperatures at pH 7.4 and limited results at pH 5 with only the results at 37°C at a number where there could be comparisons carried out. This shows there was limited diffusion at 20°C and 5°C, which represent post-mortem temperatures, for both antidepressants. The antidepressants have high pKa values with 9.4 for amitriptyline and 9.7 for nortriptyline (Baselt, 2008). This shows that at pH 7.4 both drugs would be ionised, as this pH is 2 units lower than the pKa values. The ionisation of amitriptyline and nortriptyline at both pH values were 99% and 99.5% at pH 7.4 and 99.99% and 99.99% in pH 5 respectively. More of the drugs were unionised at the higher pH. This is not preferential for diffusion across the bladder tissue membrane, which coincides with the results from the bladder studies as a small amount of the drugs diffused at all three temperatures (pH 7.4) in relation to the donor chamber concentration. The peak concentration was recorded at 6.69 mg/L and 6.69 ± 4.76 mg/L for amitriptyline and nortriptyline respectively (pH 7.4). Fewer results were obtained at pH 5, which was expected as the acidic pH would result in the basic drugs (amitriptyline and nortriptyline) becoming more ionised , 99.99% ionised for both drugs, therefore limiting the availability of the drugs to diffuse through the tissue membrane. The peak concentrations in pH 5 were 1.96 mg/L and 2.8 ± 0.32 mg/L for amitriptyline and nortriptyline respectively. However, these values were recorded from a buffer solution, which could result in a slightly different outcome than if the drugs were dissolved in urine. Furthermore, the concentrations would not constitute to an overdose purely from these results. Overall, diffusion can be seen for both rhodamine B and antidepressant drugs. The pH cannot be found as a significant factor in the diffusion of rhodamine B; however, it is a factor with amitriptyline and nortriptyline through bladder tissue within the first 100 hrs after death. In relation to other drugs the Henderson-Hasselbalch equation can be used to determine the ionisation of the drug at a particular pH and these experiments can be used as a model to determine the amount of drug that could possibly diffuse through the bladder tissue.

Tissue degradation is a component of the decomposition process and as a result was investigated in relation to drug movement from the bladder. Tissue breakdown is a physical process that would allow the release of urine from the bladder. Breakdown could be a significant factor on the concentration of drugs released if the

bladder tissue breaks down quickly, the drugs would be released at an earlier stage in the decomposition process. This could lead onto increased movement of drugs out of the bladder. The degradation was introduced by freezing bladder tissue for one week then thawing before using in the Franz cells. The comparison of fresh and degraded bladder tissue showed no significant difference in diffusion over 100 hrs in PBS at 37°C and 20°C. The lowest temperature did show increasing significant difference throughout the experiment, however, the peak concentrations were very low mostly below the limit of detection for the method. The lower pH showed no significant difference at all three temperatures. This shows that overall the tissue degradation does not affect the concentration of drugs released from the bladder tissue over approximately 100 hrs after death.

The donor chamber represents the inside of the bladder and this method allowed for the analysis of the contents of the "bladder" at the end of the experiment. This shows the movement of drugs from within the bladder to the surrounding area in a controlled environment. The donor chamber concentration decreased for all three compounds, with the largest decrease in "bladder concentration" at the highest temperature and pH. However, the difference between the original donor concentration and the final combined concentrations of the donor and acceptor chambers was very large resulting in a discrepancy in the total concentrations. The reason for this difference is most likely due to the binding of the drugs to the bladder tissue. All three of the compounds are lipophilic (with log P values of 4.92 (Hansch, Leo, 1995), 4.51 (Brodin, 1974) and 2.43 (Mah *et al.*, 2013) for amitriptyline, nortriptyline and rhodamine B respectively), which would allow them to cross cell membranes and potentially bind to cellular components.

The bladder thickness was expected to be important in diffusion across the bladder due to the Fick equation taking into account the membrane thickness. Biological tissue is variable within certain parameters and with regard to the bladder or animal therefore there will be variation in bladder thickness, dependent on the individual and also the amount of urine in the bladder. The bladder thickness range was large in this set of experiments, however the symmetrical histogram (See Figure 4.50) suggested most of the bladder thicknesses were within a small spread of values between 4.48 and 5.0 mm. In addition, the difference between fresh and degraded bladders was not significant (p 0.42) so degraded tissue does not affect bladder tissue thickness. Furthermore, the thicknesses within the same bladder also resulted in insignificant differences (p 0.23) showing there is no large variation in the thicknesses within different sections of the bladder. This could show there may not be preferential diffusion from different sections of the bladder in relation to the bladder thickness. As a result, whether a body has been found close to the time of death or within five days with differing volumes of urine present, altering the bladder membrane thickness, any drug movement through the bladder membrane, specifically amitriptyline and nortriptyline, would not be affected by these conditions.

Rhodamine B has been previously investigated in relation to permeability with a permeability coefficient determined for the isolated rabbit cornea and a cell culture using epithelium from the human cornea as a model. The permeability value from that study carried out at 35°C for rhodamine B was higher than hydrophilic drugs, $1.63 \pm 4.0 \times 10^{-5}$ cm/s, showing that the upper layers of the cornea is the urothelium

equivalent in the bladder membrane, which limits the diffusion of the hydrophilic drugs (Toropainen, Ranta and Talvitie, 2001). The two lower temperatures (20°C and 5°C) using fresh bladder sections from this research had permeability values within the same order of magnitude, with 6.8 x 10^{-5} cm/s for 20°C and 1.4 x 10^{-5} cm/s for 5°C. However, at 37°C the permeability increases by a magnitude of ten (1.8 x 10^{-4} cm/s) showing the physiological temperature increases diffusion. The antidepressant drugs (amitriptyline and nortriptyline) have been investigated in previous studies in relation to permeability, which involved diffusing amitriptyline through caco-2-cells. The permeability coefficient at 1 mM was 2.1 x 10^{-5} cm/s in pH 7.4 buffer solution at 37°C (Faassen *et al.*, 2003). This research shows consistent results with the permeability of the same order of magnitude with 1.9 x 10^{-5} cm/s, showing amitriptyline to be a highly permeable compound. In relation to nortriptyline, which is a more polar drug and the difference in pH affects the ability of the drug to penetrate a membrane due to a reduction in the amount of the non-ionised form. The permeability coefficients at pH 7.4 and 5.5 were recorded through human skin at 0.36 ± 0.04 x 10^{-6} cm/s and 0.011 ± 0.004 x 10^{-6} cm/s respectively (Melero, Garrigues and et al, 2008). The results from this research show an increase in permeability in relation to the previous study by an order of ten with permeability coefficients of 3.0 x 10^{-5} cm/s (pH 7.4) and 4.6 x 10^{-5} cm/s in pH 5. This could be due to the bladder preferentially diffusing more lipophilic drug in comparison with human skin (Scheuplein, 1970).

The permeability of all three drugs was affected only by temperature with the highest permeability at physiological temperature (37°C); this shows that peak permeability would be present close to the time of death. However, the increased permeability at the physiological temperature would not be relevant for longer post-mortem periods unless the body was found in warm climates, as the body cools to room temperature after death reducing the drug permeability independent of pH. Similar permeability values for rhodamine B were found for fresh and degraded tissue. The permeability values were 1.8 x 10^{-4} cm/s (pH 7.4) and 1.8 x 10^{-4} cm/s (pH 5) for fresh tissue and 3.6 x 10^{-4} cm/s (pH 7.4) and 1.4 x 10^{-4} cm/s (pH 5) for degraded tissue at 37°C. These results show that if the bladder was degraded due to decomposition this would not significantly affect the permeability of rhodamine B. This degradation would occur in the later stages of the decomposition process and even with the results showing higher concentrations of rhodamine B at physiological temperature with degraded bladders, with peak concentrations at 1.70 ± 1.0 mg/L for fresh tissue and 2.63 ± 2.46 mg/L for degraded tissue, this scenario would not be realistic. This is due to the body being reduced to room temperature before significant tissue degradation could occur. At room temperature, the rhodamine B permeability has decreased by a magnitude of ten for both fresh (6.8 x 10^{-5} cm/s (pH 7.4)) and degraded bladder tissues (5.8 x 10^{-5} cm/s (pH 7.4)). Within the same bladder, the results show if the body remained at physiological pH the permeability would remain constant until the body cooled to room temperature with 3.0 x 10^{-4} cm/s at 37°C and 3.5 x 10^{-4} cm/s at 20°C. However, this would not occur due to the cessation of the aerobic respiration and the commencement of anaerobic respiration that produces acidic compounds after death. The more acidic pH results show the permeability is lower at room temperature than pH 7.4 and stays constant at this value even when the body would be stored in the morgue fridge before autopsy. This could be the result of the drugs becoming more ionised at the lower pH, which would limit the movement of the drugs across the biological tissue.

The permeability of the antidepressant drugs has similar results of the same magnitude with slightly higher permeability of amitriptyline than nortriptyline. The trend in permeability is similar to rhodamine B with decreasing permeability with decreasing temperature. This shows that as the body cools there is less diffusion of both amitriptyline and nortriptyline from the bladder at physiological pH. The lower permeability values for the physiological temperature could be due to the non-steady state results. The comparison of permeability values show amitriptyline has the highest permeability, then rhodamine B and finally nortriptyline with 9.7×10^{-5} cm/s, 6.58×10^{-5} cm/s and 3.0×10^{-5} cm/s respectively (pH 7.4) at 20°C. This shows no trend involving the molecular weight, log p or pKa values as amitriptyline (MW: 277.4 g/mol, log P: 4.92, pKa: 9.4) (Hansch, Leo, 1995; Baselt, 2008; Negrusz, 2013) and nortriptyline (MW: 263.4 g/mol, log P: 4.51, pKa: 9.7) (Brodin, 1974; Baselt, 2008; Negrusz, 2013) have similar values and rhodamine B (MW: 479 g/mol, log P: 2.43, pKa: 4.2) (Zhang *et al.*, 2011; Mah *et al.*, 2013; Qi, Gao and Zhang, 2013) has a higher molecular weight and lower values for log P and pKa. This result was not expected, as the drugs with similar characteristics would have similar permeability results however, all three of the peak permeability values are of the same magnitude showing the environmental conditions have a larger effect on the alteration of the magnitude of the permeability.

The permeability coefficients of each drug could be used in further work involving calculating the diffusion coefficient, which could then be used to determine the time taken for each drug to diffuse to set distances (Clark, Edeson and Ryall, 1983). This would be useful in relation to calculating the time taken for a drug to reach the femoral vein from the bladder, measurements between the bladder and the femoral vein would be needed for these equations.

The pH change in the acceptor chambers was consistent with the knowledge that the physiological pH decreases overtime after death with pH 6.87 ± 0.24 at the end of the pH 7.4 fresh inter-bladder experiment at 37°C. The lower pH that is achieved after the first stages of autolysis increases during the later stages of decomposition with the final acceptor chamber pH of pH 6.95 ± 0.16 from the pH 5 fresh inter-bladder experiment at 37°C. This shows that pH values *in vivo* can be replicated *in vitro*.

Overall, the movement of rhodamine B, amitriptyline, and nortriptyline from the bladder has been shown at physiological temperature and pH. The length of the experiments mimicked the first five days after death and the results show that there is selective diffusion over this time dependant on the environmental conditions. The optimal conditions for the movement of drugs after death include physiological conditions in relation to both temperature (37°C) and pH (pH 7.4). The diffusion was hindered the most by the mortuary fridge temperature (5°C) and post-mortem pH (pH 5). Even with higher drug concentrations found with amitriptyline and nortriptyline, there would be dilution of the drugs that is likely to occur when diffusing through the tissues and a larger volume of fluid in the peritoneal cavity after the drugs have left the bladder. Furthermore, constant temperatures would not be maintained after death, especially physiological conditions. Therefore, the diffusion would be based on the two lower post-mortem temperatures resulting in very limited diffusion of drugs from the bladder with average peak cumulative concentrations of 2.73 ± 2.31 mg/L and 0.85 ± 0.89 mg/L at 20°C, also 0.54 ± 0.46 mg/L and 0.31 ± 0.49 mg/L at 5°C in PBS and AA respectively.

These results show that there would not be an issue if the deceased was autopsied and toxicological samples taken up to five days after death. This is due to the small cumulative concentrations found in the acceptor chamber for both sets of drugs, rhodamine B and the antidepressant drugs, with the peak concentration of 6.69 ± 4.76 mg/L for nortriptyline in pH 7.4 at 37°C.

This concludes varying diffusion dependant on environmental conditions with the most diffusion using physiological conditions, based on the model created by diffusing rhodamine B and two antidepressant drugs through bladder sections, in relation to affecting femoral vein blood samples taken at autopsy for drug concentration change. To create a more realistic model whole bladders were used to determine the amount of rhodamine B released post-mortem.

Chapter 5 – Diffusion of Drugs from the Whole Bladder

5.1 Introduction

Previous work has suggested that there is a possibility of drug diffusion from the bladder to the femoral vein. If verified this could lead to a misinterpretation of femoral blood drug concentrations in forensic cases (Moriya and Hashimoto, 2001). The previous chapter focussed on the diffusion of chemicals (rhodamine B, amitriptyline, and nortriptyline) across sections of bladder to determine if there is variation in the rate and extent of drug diffusion between different bladders and within the same bladder. However, this is not able to mimic real casework as in most cases the bladders are intact and would be storing urine possibly containing drugs.

The bladder is a storage organ and during life, the bladder holds a limited amount of urine, 351.3 ± 86.0 ml for men and 325.0 ± 76.7 ml for women (Kanyilmaz, Calis, Cinar, 2013), before it is voided. However, in some post-mortem cases death occurs before this is possible, which results in a bladder filled with urine possibly containing drugs still present in a body that starts to decay almost immediately after death. There has been studies relating urinary retention to certain drug classes including antipsychotics (Bozikas, Petrikis and Karavatos, 2001), benzodiazepines (Benazzi, 1998) and tricyclic antidepressants (Remick, 1988) mainly thought to be due to the anticholinergic actions of these drugs. In addition, a CT-based study suggested links between the volume in the bladder and intoxication with results showing there is a correlation between the calculated volume of the bladder and positive results for drugs in the toxicology report. A number of drugs were included in the study including alcohol, cocaine, opiates and also poly-drug variations. The results showed fatally intoxicated individuals had higher bladder volumes than non-fatally intoxicated individuals. Therefore, fatal intoxication of these drugs result in urinary retention (Rohner and Franckenberg, 2013). Different areas within the body start to decompose at different times and rates; this is partly the reason for preferential sampling sites. In addition, peripheral sites are less affected by PMR including the femoral vein. However, with limited data on the degradation of the intact bladder within the body it is not known when this organ begins to decay and release the entire contents into abdominal cavity, rather than releasing drugs via diffusion. If this occurs at the early stages of decomposition the drug laden urine could be released allowing the drugs free to diffuse into possible sampling sites including the femoral vein.

The bladder has been assumed impenetrable; however, research has shown that numerous compounds can diffuse through the bladder including barbiturates, basic drugs (atropine, neostigmine and physotigmine) (Borzelleca, 1959) and nicotine (Borzelleca, 1963). In living tissue there are active transport mechanisms that transport sodium (Wickham, 1964; Diamond, 1976) into the bladder membrane. Passive permeability of substances is very selective however, urea (Maffly *et al.*, 1960) has been shown to passively diffuse through the bladder membrane, increasing the permeability with the use of hormones. This shows that passive diffusion from the bladder is possible and the bladder is not impenetrable to all substances in life, shown by lipophilic drugs used in chemotherapy treatments, thiotepa and mitomycin C, with 20-97% and 0-80% bioavailability of the drugs from the bladder respectively (Czech, 1971; Dalton, Wientjes, 1991).

It has been shown that treating mainly superficial bladder cancers with intravesical therapy, directly into the bladder using catheterisation, can result in 1000 times higher drug concentration in the bladder than if treated

via plasma. Therefore, this has lead onto research into avenues that increase the permeability of the bladder wall to certain drugs to treat disease, including bladder cancer (Wientjes *et al.*, 1991). More specifically, increasing permeability of the affected area within the bladder membrane to the drugs. This is usually superficial bladder cancer, and helps to reduce the toxic effects of the chemotherapeutic drugs to healthy cells (Grabnar *et al.*, 2006; Allison, Sadiq, Baronou, 2017). Introducing the hydrophilic drugs to the bladder membrane via the intravesical method allows for longer contact with the tumour and more penetration into the tissue. The increased penetration is due to the shedding of the urothelium cells, otherwise known as desquamation (Grabnar *et al.*, 2006).

The urothelium has been shown to be damaged and lost in the early post-mortem stages (Jost, Gosling and Dixon, 1989); this could result in a natural increase in permeability after death. It has been demonstrated that bladder tissue samples taken later than 12 hrs after death have lost this top layer within the bladder lumen, leaving less mature cells that could allow for an increase in the passive movement of substances (Newman, 1981). The increase in permeability has been shown to be possible through damage (Lavelle *et al.*, 1998, 2002). The alteration of absorption due to damaged and distended bladders was demonstrated in a study that used both human and animal models. Two sugars (4% lactulose - 1% rhamnose solution) were inserted into a set of intact and damaged bladders and blood samples were taken 30 minutes later. Neither sugar was detected in the blood samples from the subjects with intact bladders. However, both sugars were detected and quantified in the blood samples of subjects with damaged and distended bladders (Erickson *et al.*, 2000).

The concentration of drugs and the volume of urine have also shown to possibly effect drug diffusion through bladder tissue. A short-term study showed the process of drug movement out of the bladder is as expected a passive process. This study used rabbit bladders and investigated the effect of different concentrations and volumes of nicotine. The range of volumes added to the bladder was between 1 – 18 ml, which could be sufficient to reduce the thickness of the bladder walls for diffusion. The results show that the increase in volume decreased the diffusion of drug. Also, changing the concentration did affect the diffusion rate until the possible saturation at the highest concentration, which was the reason for the determination of a passive diffusion process (Borzelleca and Lowenthal, 1967). This would be expected as the rate of diffusion is usually determined by the magnitude of difference in concentration between the two sides of the membrane. This shows concentration is the rate-limiting parameter in that diffusion process.

5.2 Aims

The aim of this chapter is to determine if the intact bladder breaks down in the same way as the bladder sections and also if the rate of diffusion is changed. As with chapter 4 particular attention will be paid to the effect of concentration, pH and bladder volume on the diffusion of rhodamine B. These experiments were carried out at 20°C, which is used as room temperature that a body would equilibrate to after death.

5.3 Results

The experiments were based around looking into the effect on the amount of rhodamine B diffusing through whole porcine bladders dependant on some of the incorporated conditions from chapter 4. The length of the experiment was over 5 days to mimic the previous experimental design with one sample taken per day. The comparisons included solution pH, rhodamine B concentration, and bladder volume. A number of experiments were carried out using whole bladders that were filled with the model compound (rhodamine B) and control solutions (pH 7.4 and 5). These experiments were carried out to compare the results to the bladder section experiments (See Chapter 4) to determine if there are consistent results in regards to drug diffusion. The main parameters that were investigated included comparing two values for concentration (100 mg/L and 200 mg/L) of the drugs, volume (full and half-filled bladder), and solution pH (7.4 and 5). The experiments were carried out at 20°C. The control experiments used bladders filled with blank solution (pH 7.4 and 5). The results of the blank solutions had no significant effect on the rhodamine B results with average results below all the rhodamine B bladder study results (Data not shown).

5.3.1 Influence of concentration on the amount of rhodamine B diffusing through whole bladders

Two concentrations of rhodamine B, 100 mg/L and 200 mg/L, were used to determine the influence of concentration on the amount of rhodamine B diffusing through fresh bladders using both full and half-filled bladders at both pH 7.4 and pH 5. As can be seen in Figure 5.1 to Figure 5.4 the highest cumulative concentration of rhodamine B was observed when the bladder was full with buffer solution at pH 7.4 and the rhodamine B was at a concentration of 100 mg/L. The peak cumulative concentration of rhodamine B was 3.5 ± 1.02 mg/L at 97 ± 0.03 hrs post-mortem. The lowest cumulative concentration of rhodamine B was observed in pH 5 buffer solution with a full bladder and rhodamine B at a concentration of 100 mg/L, with the concentrations < the 0.156 mg/L limit of detection (See Figure 5.3).

A significant difference in cumulative rhodamine B concentration was determined between the two rhodamine B concentrations (100 mg/L and 200 mg/L) at 99 ± 0.02 hrs in full bladders at pH 7.4 buffer solution with more diffusion from 100 mg/L rhodamine B ($p < 0.0001$) (See Figure 5.5). The peak cumulative rhodamine B concentration at 100 mg/L was 3.5 ± 1.02 mg/L and at 200 mg/L was 1.23 ± 0.26 mg/L (See Figure 5.1). The significant difference between the two rhodamine B concentrations (100 mg/L and 200 mg/L) at pH 5 using full bladders started at 50 ± 0.02 hrs and increased at 99 ± 0.02 hrs with increased diffusion from 200 mg/L rhodamine B (p 0.05 and < 0.0001) (See Figure 5.7). The cumulative concentrations of rhodamine B (pH 5) using full bladders at 99 ± 0.02 hrs were 0.58 ± 0.95 mg/L at 200 mg/L and a concentration below the limit of detection at 100 mg/L (See Figure 5.3).

5.3.1.1 pH comparison of rhodamine B for whole bladders

The pH showed a visual difference with higher cumulative rhodamine B concentrations at pH 7.4 in comparison with pH 5. The peak cumulative rhodamine B concentration at pH 7.4 was 3.5 ± 1.02 mg/L at 100 mg/L rhodamine B and at pH 5 was 0.67 ± 0.89 mg/L for 100 mg/L rhodamine B 97 ± 0.03 hrs. In addition, the concentration shows more of a difference with 100 mg/L resulting in higher cumulative concentrations of

rhodamine B (pH 7.4) (See Figure 5.1). However, at pH 5 the 200 mg/L rhodamine B results in higher cumulative concentrations than 100 mg/L (See Figure 5.3). The peak concentrations were 0.58 ± 0.95 mg/L at 200 mg/L and at 100 mg/L a concentration below the limit of detection at 73 ± 0.01 hrs. This shows lowering the pH results in requiring higher rhodamine B concentrations to diffuse through the tissue and still resulting in lower results than at PBS pH 7.4.

5.3.1.2 Bladder volume comparison of rhodamine B for whole bladders

In addition, the bladder volume was altered using both full and half-filled bladders. The volume only affected the cumulative concentrations of rhodamine B at PBS pH 7.4, with a noteworthy increase in the cumulative concentration for 100 mg/L rhodamine B in the full bladder (See Figure 5.1 and 5.2). The half-filled bladders at pH 7.4 had negligible differences in rhodamine B cumulative concentration between the two concentrations (100 mg/L and 200 mg/L). The peak cumulative concentrations for the half-filled bladders at pH 7.4 were 0.96 ± 0.73 mg/L for 100 mg/L at 73 ± 0.01 hrs and 0.92 ± 0.22 mg/L for 200 mg/L rhodamine B at 96 ± 0.01 hrs (See Figure 5.2). The rhodamine B diffusion in the full bladders at pH 5 had major differences from 50 ± 0.02 hrs (p 0.05) (See Figure 5.7). However, the half-filled bladders at pH 5 showed minimal differences in the cumulative concentrations of rhodamine B (See Figure 5.8). The peak cumulative concentrations were 0.67 ± 0.89 mg/L for 100 mg/L at 97 ± 0.03 hrs and 0.3 ± 0.1 mg/L for 200 mg/L rhodamine B at 25 ± 0.01 hrs.

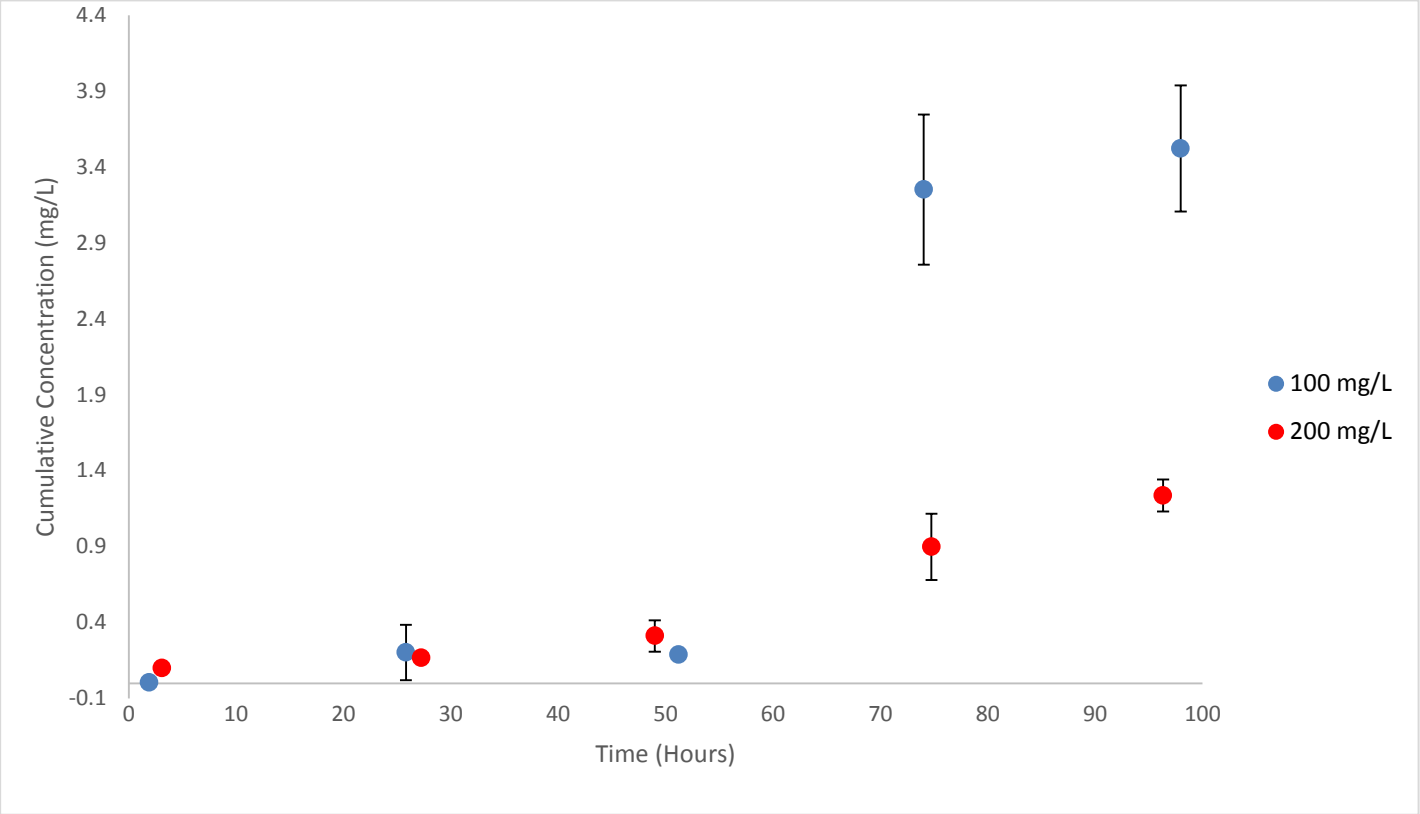

Figure 5.1 Average rhodamine B cumulative concentration using six fresh whole bladders, comparison at 100 mg/L and 200 mg/L with full bladder volume at pH 7.4 (n=6) (Error bars are ± S.D.)

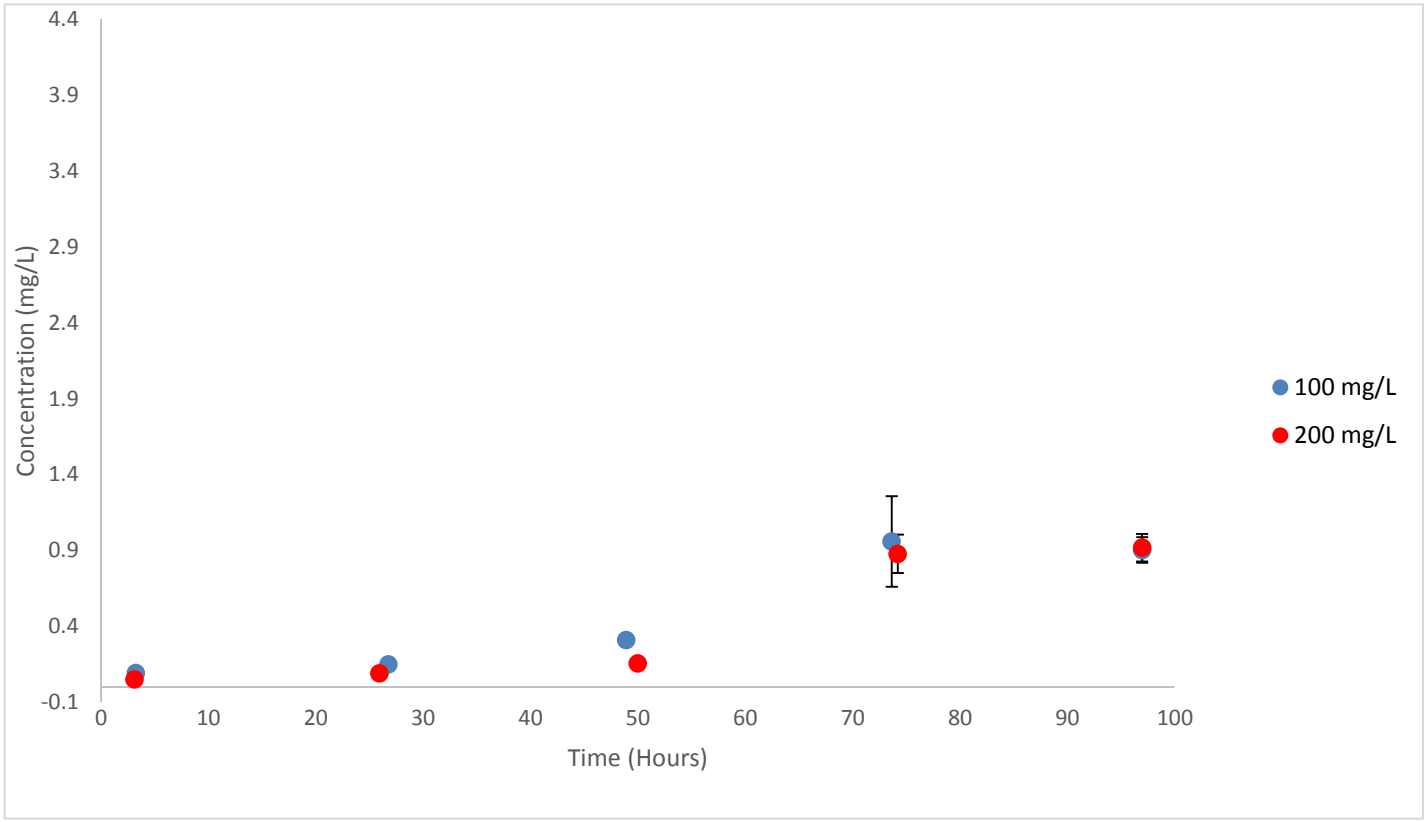

Figure 5.2 Average rhodamine B cumulative concentration using six fresh whole bladders, comparison at 100 mg/L and 200 mg/L with half-full bladder volume at pH 7.4 (n=6) (Error bars are ± S.D.)

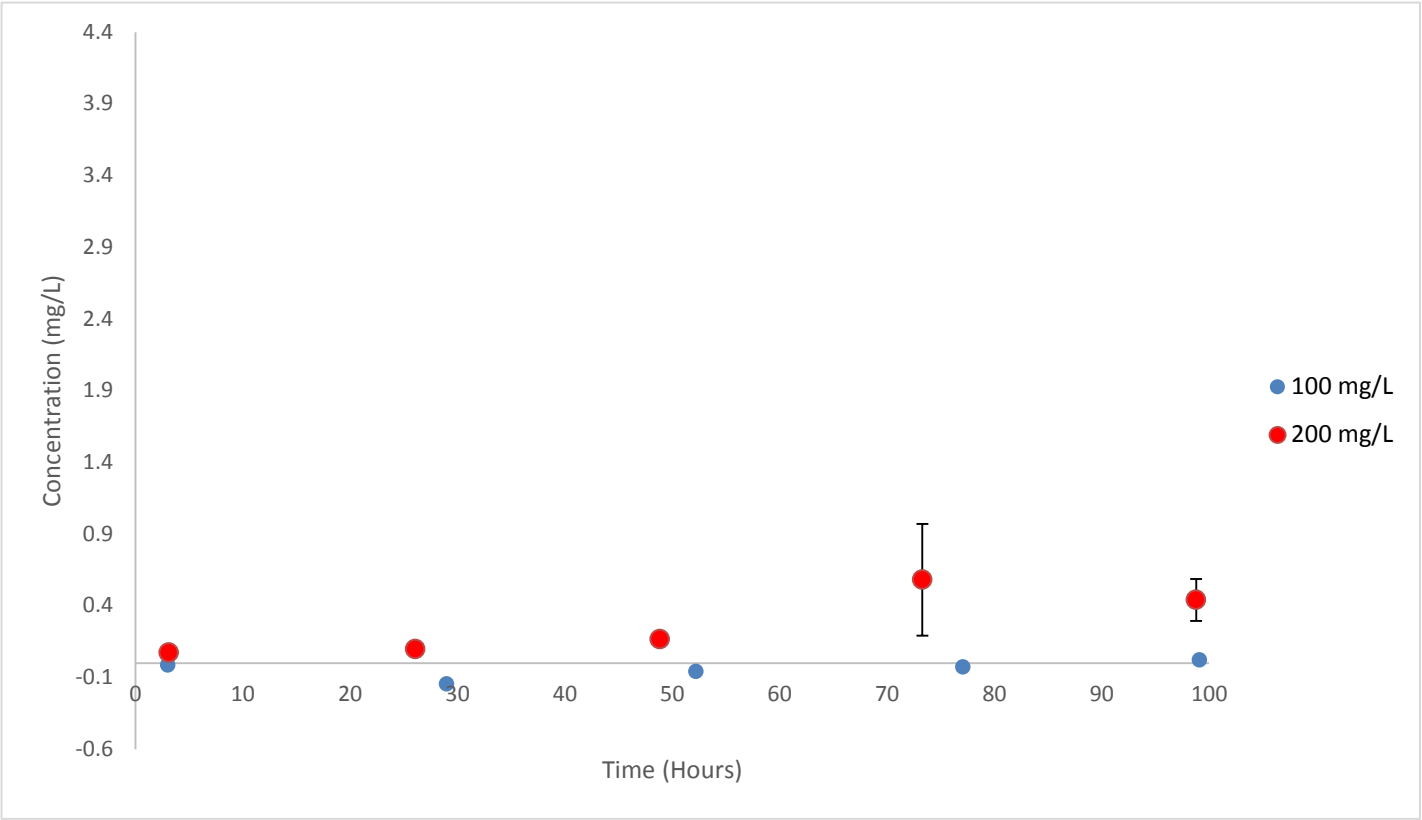

Figure 5.3 Average rhodamine B cumulative concentration using six fresh whole bladders, comparison at 100 mg/L and 200 mg/L with full bladder volume at pH 5 (n=6) (Error bars are ± S.D.)

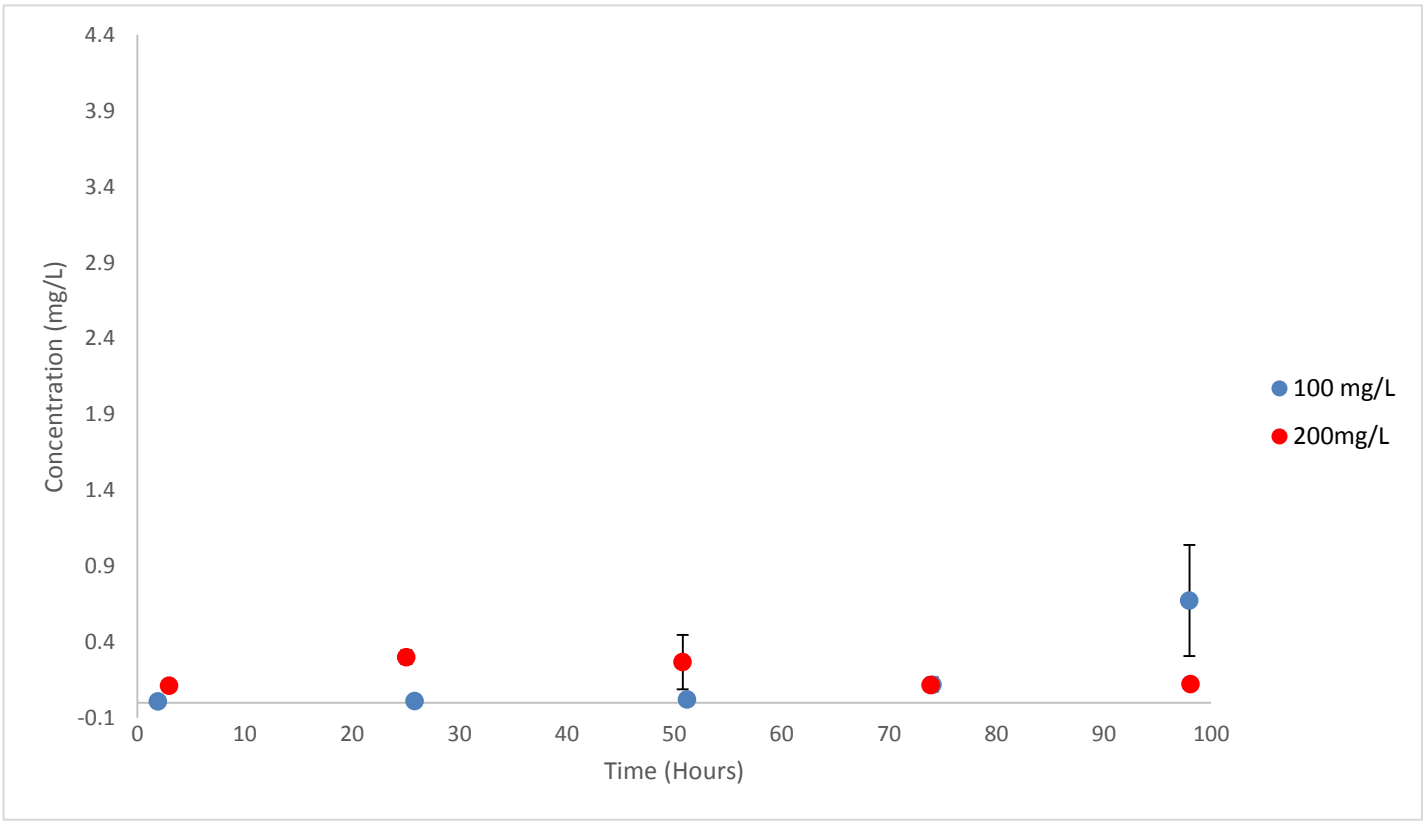

Figure 5.4 Average rhodamine B cumulative concentration using six fresh whole bladders, comparison at 100 mg/L and 200 mg/L with half-full bladder volume at pH 5 (n=6) (Error bars are ± S.D.)

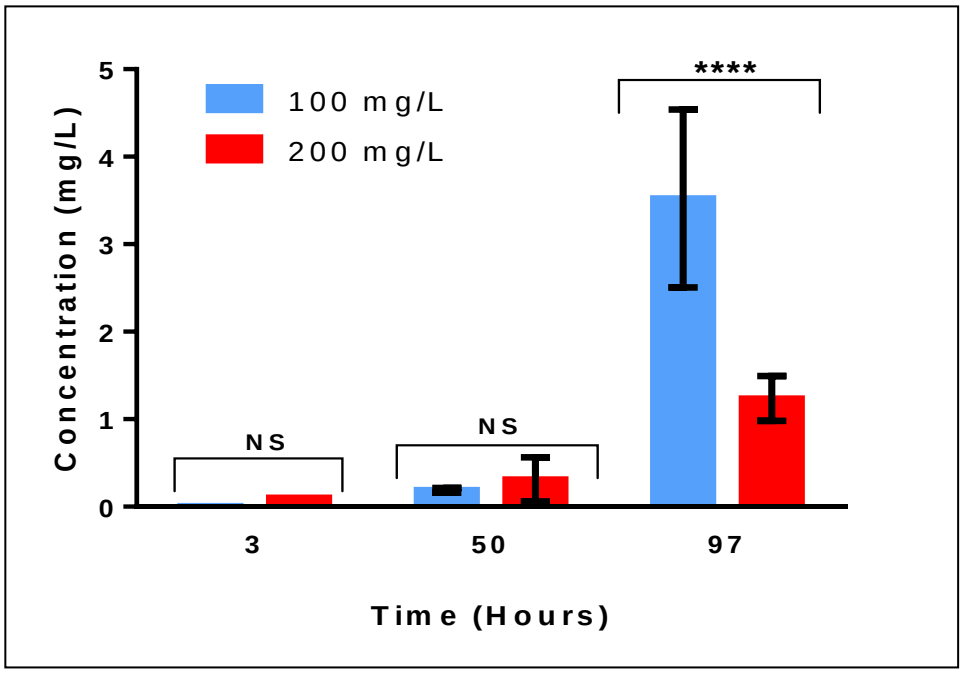

Figure 5.5 Two-Way ANOVA results of the comparison of rhodamine B concentration (100 mg/L and 200 mg/L) in six fresh whole bladders at full volume (pH 7.4) over approximately 100 hrs (Error bars are ± S.D.)

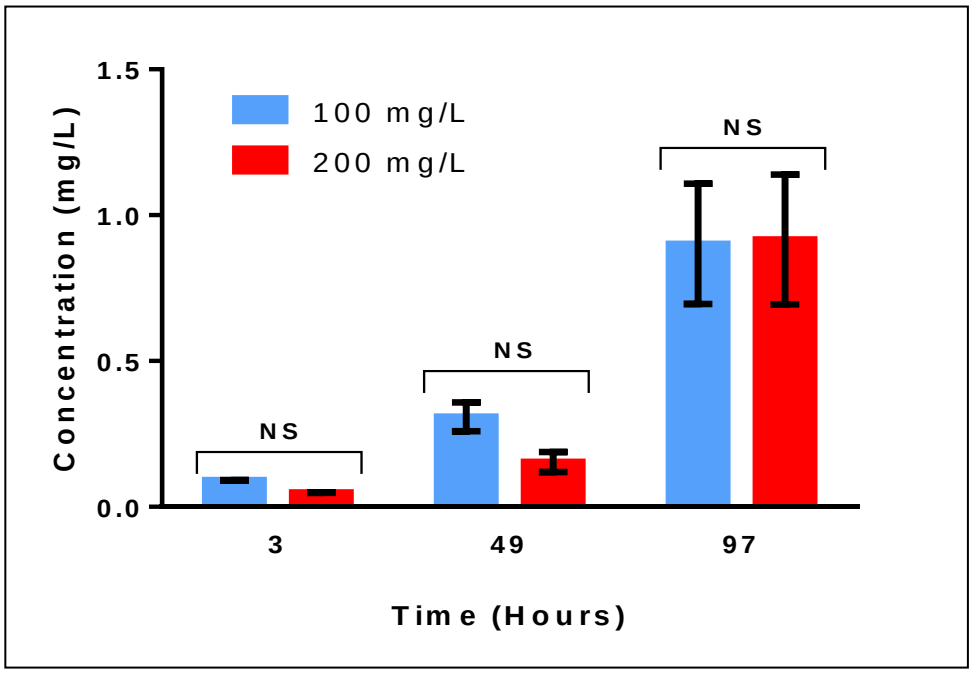

Figure 5.6 Two-Way ANOVA results of the comparison of rhodamine B concentration (100 mg/L and 200 mg/L) in six fresh whole bladder at half-full volume (pH 7.4) over approximately 100 hrs (Error bars are ± S.D.)

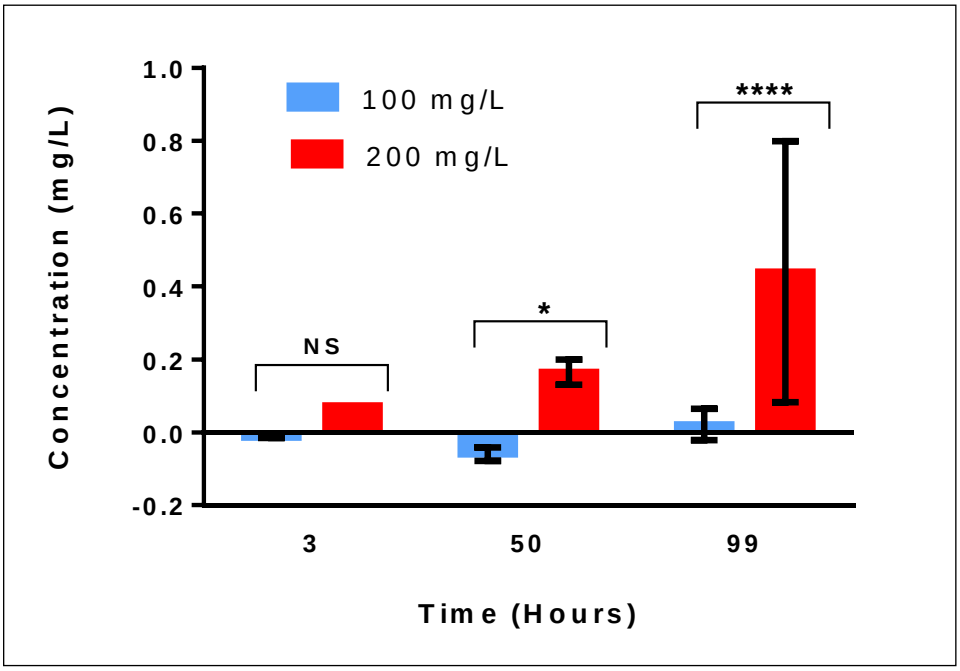

Figure 5.7 Two-Way ANOVA results of the comparison of rhodamine B concentration (100 mg/L and 200 mg/L) in six fresh whole bladders at full volume (pH 5) over approximately 100 hrs (Error bars are ± S.D.)

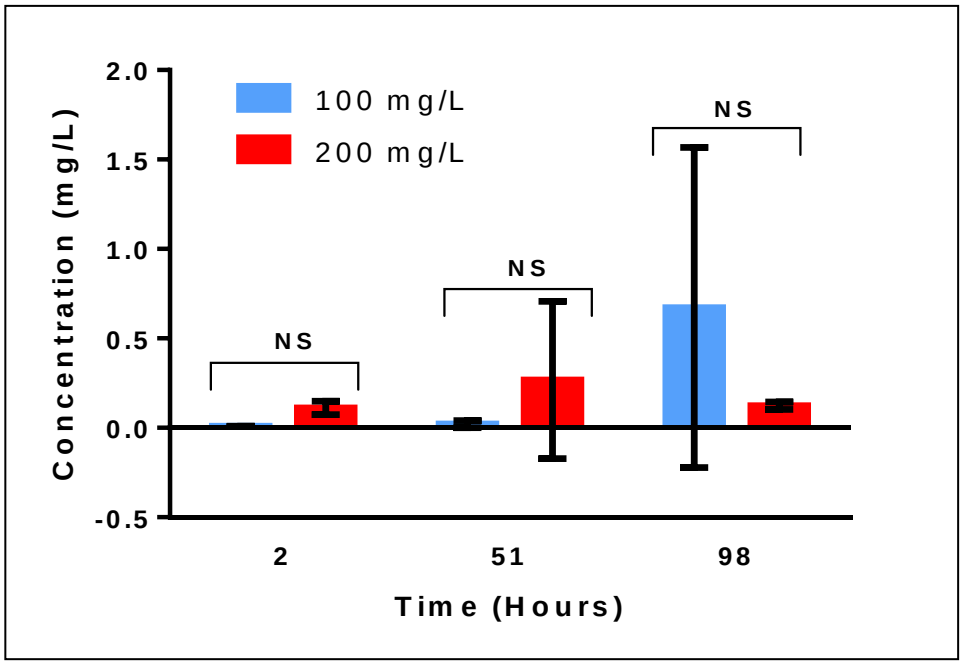

Figure 5.8 Two-Way ANOVA results of the comparison of rhodamine B concentration (100 mg/L and 200 mg/L) in six fresh whole bladders at half-full volume (pH 5) over approximately 100 hrs (Error bars are ± S.D.)

5.3.2 Influence of bladder volume on the amount of rhodamine B diffusing through whole bladders

In order to investigate the effect the volume within the bladder had on the amount of rhodamine B diffusing through whole bladders, two volumes of rhodamine B were used that included full bladders and half-full bladders. A fresh set of calibration standards was prepared each day for each bladder experiment using the method from section 2.1.5.1 using pH 7.4 and pH 5. The two rhodamine B concentrations (100 mg/L and 200 mg/L) were also investigated in pH 5 and pH 7.4 over 5 days.

Figure 5.9 – 5.12 shows the highest cumulative concentration of rhodamine B was observed with a full bladder at pH 7.4 with the lower concentration of 100 mg/L of rhodamine B at 3.5 ± 1.02 mg/L at 97 ± 0.03 hrs (See Figure 5.9). The other three sets of parameters (200 mg/L at pH 7.4 / 100 mg/L at pH 5 / 200 mg/L at pH 5) show minimal drug diffusion throughout the experiment regardless of the volume in the bladder throughout the duration of the experiment (See Figures 5.10 – 5.12). The lowest cumulative concentration of rhodamine B was observed in pH 5 buffer solution with a full bladder and rhodamine B at a concentration of 100 mg/L, with the concentrations < the 0.156 mg/L limit of detection (See Figure 5.11).

There was significant difference between the cumulative rhodamine B concentrations in relation to bladder volume (pH 7.4) using both 100 mg/L and 200 mg/L rhodamine B solution at 97 ± 0.03 hrs and 96 ± 0.01 hrs respectively with more diffusion from the full bladder ($p < 0.0001$ and 0.01) (See Figures 5.13 and 5.14). The peak cumulative concentrations of rhodamine B were 3.5 ± 1.02 mg/L for the full bladders and 0.95 ± 0.73 mg/L for half-filled bladders using 100 mg/L rhodamine B at 97 ± 0.03 hrs (See Figure 5.13). The significant difference decreased for the higher rhodamine B concentration of 200 mg/L (p 0.01) (See Figure 5.14) rhodamine B in comparison with 100 mg/L ($p < 0.0001$) (See Figure 5.13). The peak cumulative concentrations were 1.24 ± 0.26 mg/L for full bladders and 0.91 ± 0.22 mg/L for half-filled bladders using 200 mg/L rhodamine B at 96 ± 0.01 hrs (See Figure 5.14). There was no significant difference in volumes at pH 5. The peak cumulative concentrations between the two volumes were 0.67 ± 0.89 mg/L at 97 ± 0.03 hrs in half-filled bladders at 100 mg/L and 0.58 ± 0.36 mg/L using 200 mg/L rhodamine B at 73 ± 0.01 hrs (See Figures 5.15 and 5.16).

5.3.2.1 pH comparison of rhodamine B for whole bladders

The pH showed higher cumulative rhodamine B concentrations at pH 7.4 in comparison with pH 5. The peak cumulative rhodamine B concentration at pH 7.4 was 3.5 ± 1.02 mg/L at 100 mg/L rhodamine B (See Figure 5.9) and at pH 5 was 0.67 ± 0.89 mg/L for 100 mg/L rhodamine B 97 ± 0.03 hrs (See Figure 5.11). In addition, the results from the different initial rhodamine B concentrations were affected with 100 mg/L resulting in higher cumulative concentrations of rhodamine B (pH 7.4). The peak cumulative concentrations were 3.5 ± 1.02 mg/L in comparison with 1.24 ± 0.26 mg/L at 200 mg/L rhodamine B (See Figures 5.9 and 5.11). However, at pH 5 the 200 mg/L rhodamine B results in higher cumulative concentrations than 100 mg/L. The peak cumulative concentration was 0.67 ± 0.89 mg/L at 100 mg/L in comparison with 0.58 ± 0.36 mg/L at 200 mg/L rhodamine B (See Figures 5.11 and 5.12). This shows at a lower pH a higher concentration of

rhodamine B is required for diffusion to occur, and that still achieves lower cumulative rhodamine B concentrations than at PBS pH 7.4.

5.3.2.2 Concentration comparison of rhodamine B for whole bladders

The comparison of the two rhodamine B concentrations resulted in higher cumulative concentrations at 100 mg/L (pH 7.4) in comparison to 200 mg/L. The peak cumulative rhodamine B concentrations were 3.5 ± 1.02 mg/L at 97 ± 0.03 hrs for 100 mg/L (See Figure 5.9) and 1.24 ± 0.26 mg/L at 96 ± 0.01 hrs (See Figure 5.10) for 200 mg/L both in full bladders. Consistently, slightly higher results were found at 100 mg/L (pH 5) against 200 mg/L however, these cumulative concentrations were still lower than at PBS pH 7.4. The peak cumulative rhodamine B concentrations were 0.67 ± 0.89 mg/L at 97 ± 0.03 hrs (See Figure 5.11) for 100 mg/L using half-filled bladders and 0.58 ± 0.36 mg/L at 73 ± 0.01 hrs for 200 mg/L (See Figure 5.12) in full bladders.

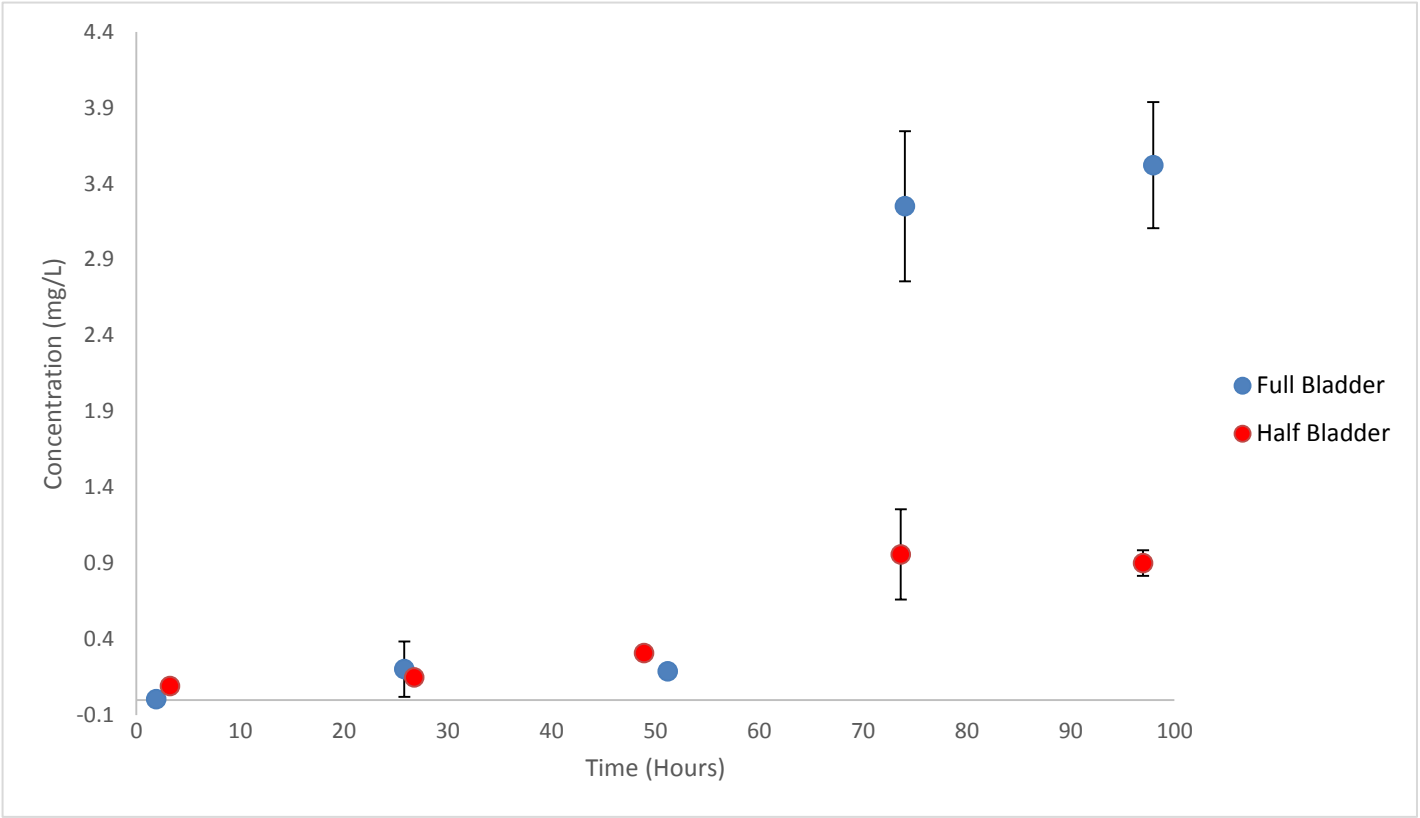

Figure 5.9 Average rhodamine B cumulative concentration using six fresh whole bladders, comparison using 100 mg/L rhodamine B in six full and half-filled bladders at pH 7.4 (n=6) (Error bars are ± S.D.)

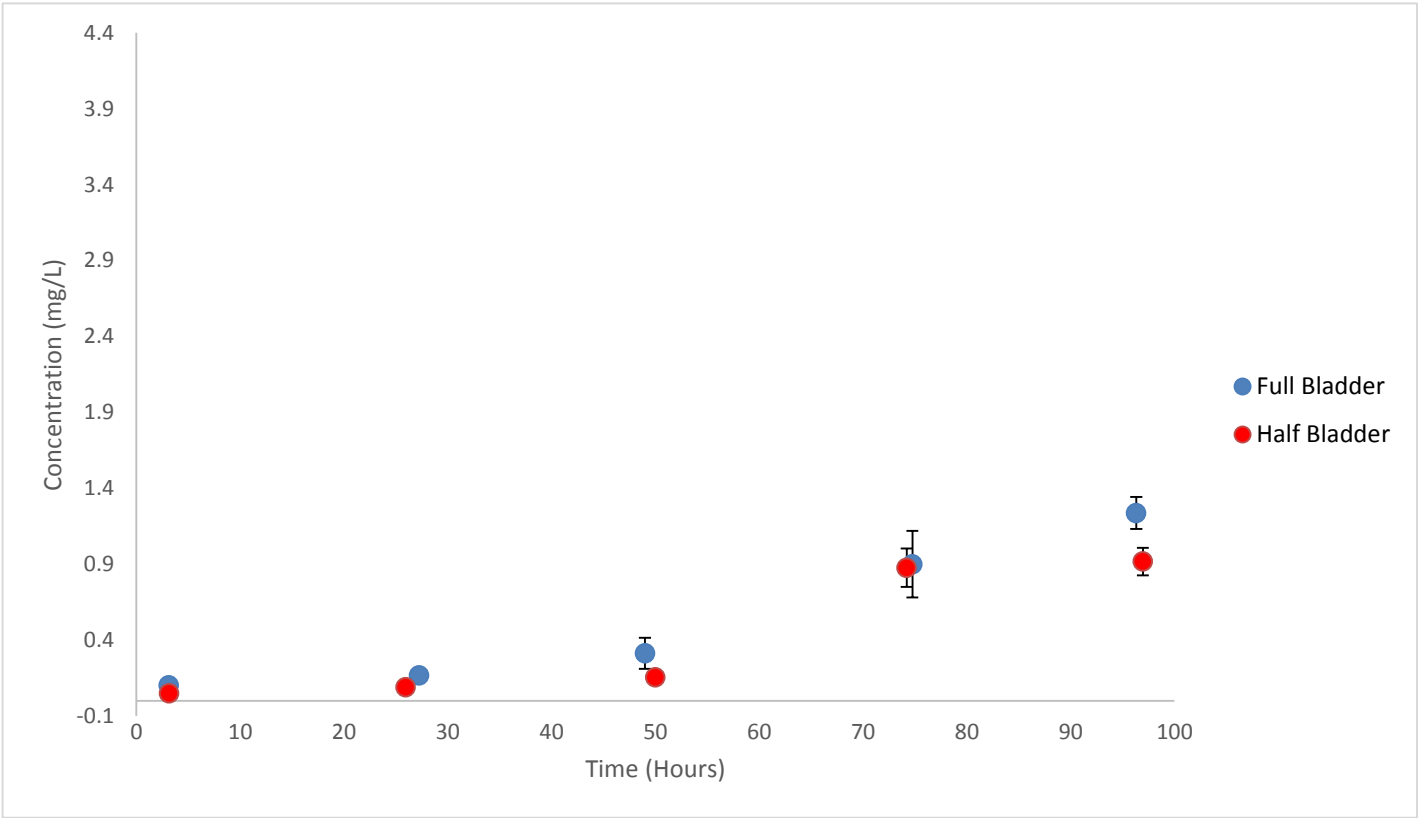

Figure 5.10 Average rhodamine B cumulative concentration using six fresh whole bladders, comparison using 200 mg/L rhodamine B in six full and half-filled bladders at pH 7.4 (n=6) (Error bars are ± S.D.)

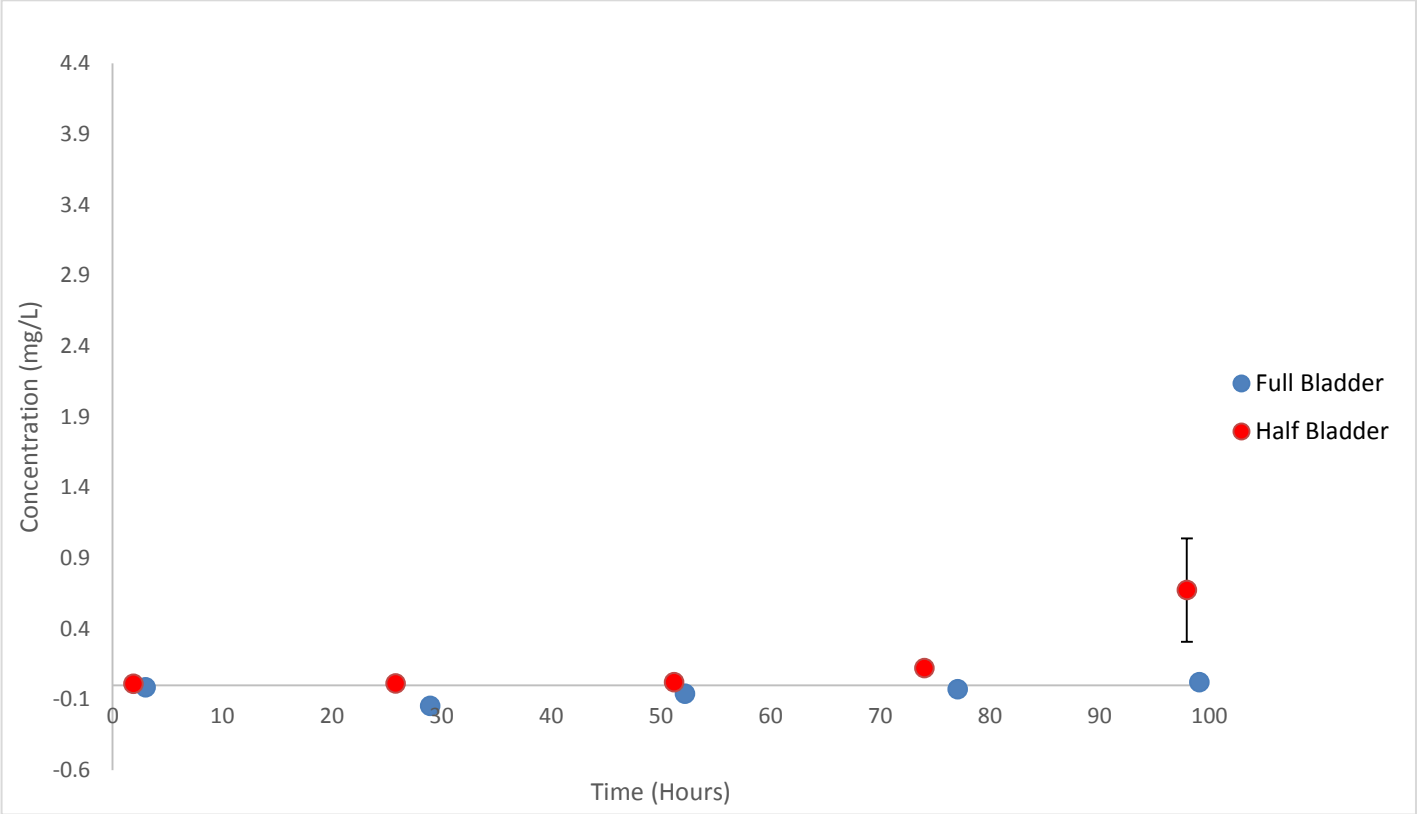

Figure 5.11 Average rhodamine B cumulative concentration using six fresh whole bladders, comparison using 100 mg/L rhodamine B in six full and half-filled bladders at pH 5 (n=6) (Error bars are ± S.D.)

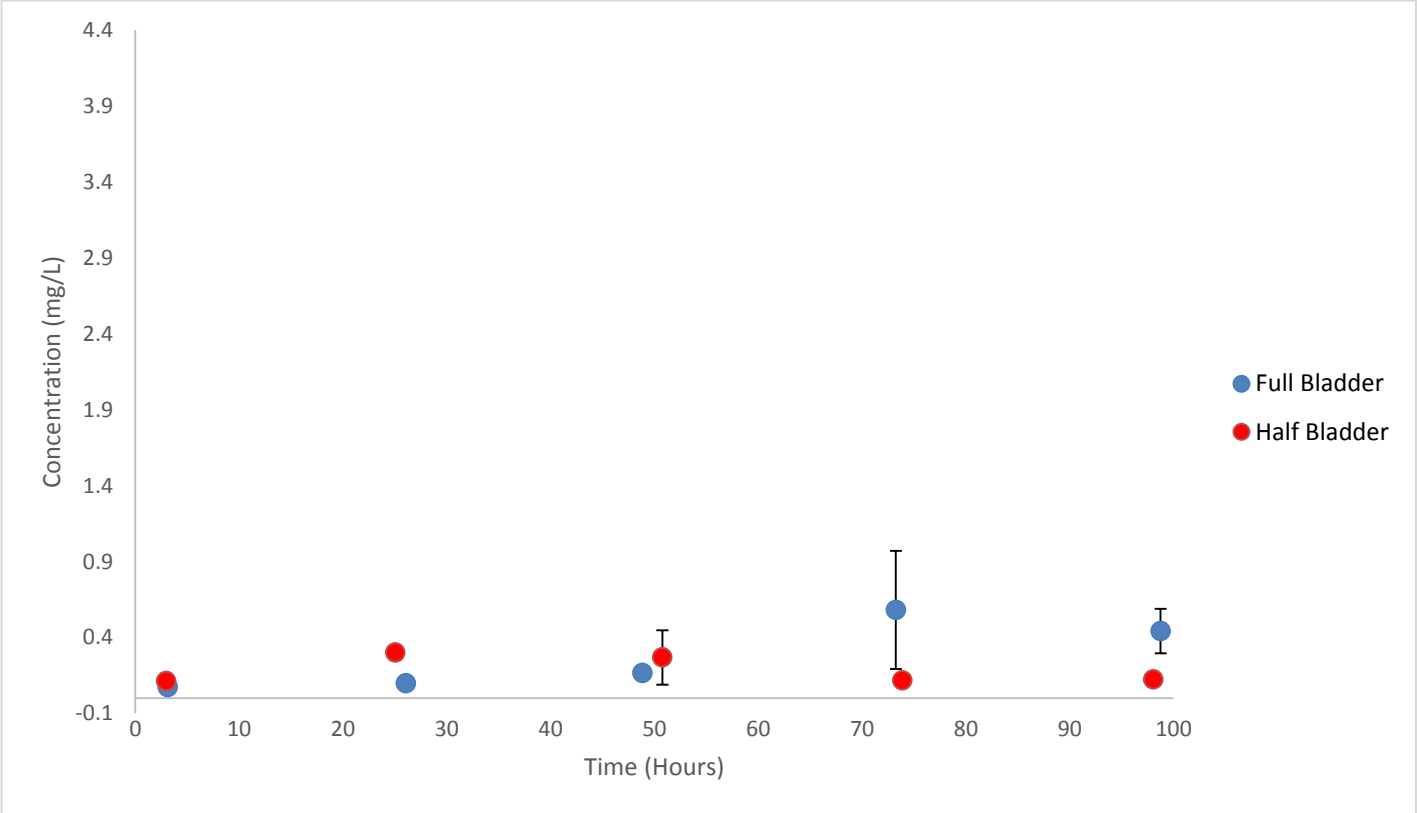

Figure 5.12 Average rhodamine B cumulative concentration using six fresh whole bladders, comparison using 200 mg/L rhodamine B in six full and half-filled bladders at pH 5 (n=6) (Error bars are ± S.D.)

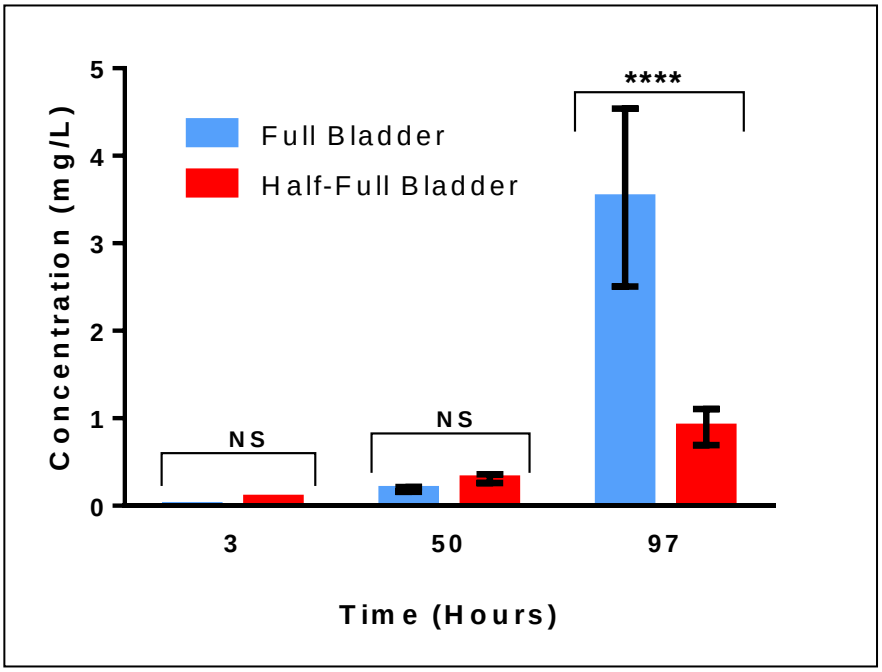

Figure 5.13 Two-Way ANOVA results of rhodamine B concentration in relation to the comparison of bladder volume (full and half-filled) of 100 mg/L rhodamine B in six fresh whole bladders at pH 7.4 over approximately 100 hrs (Error bars are ± S.D.)

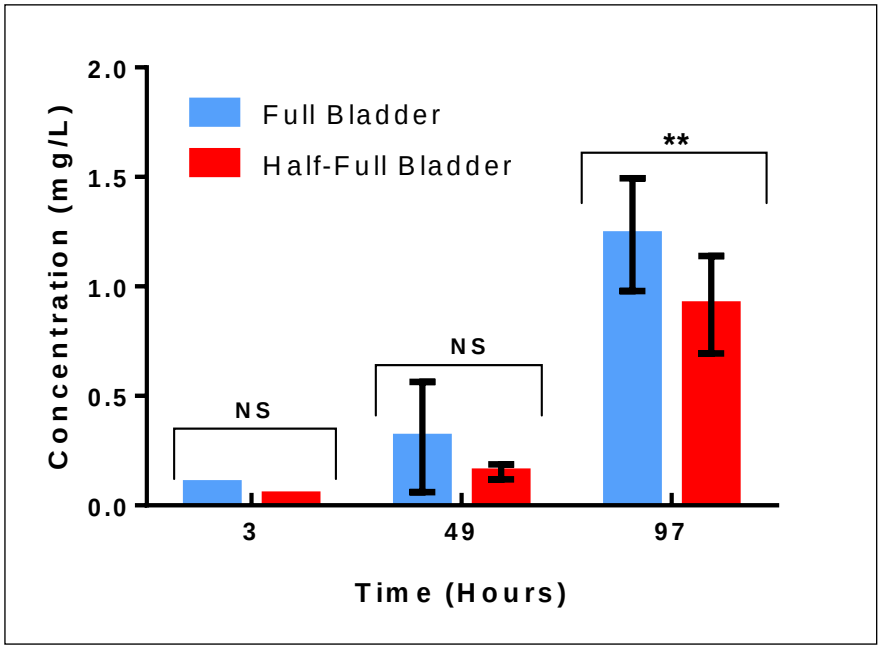

Figure 5.14 Two-Way ANOVA results of rhodamine B concentration in relation to the comparison of bladder volume (full and half-filled) of 200 mg/L rhodamine B in six fresh whole bladders at pH 7.4 over approximately 100 hrs (Error bars are ± S.D.)

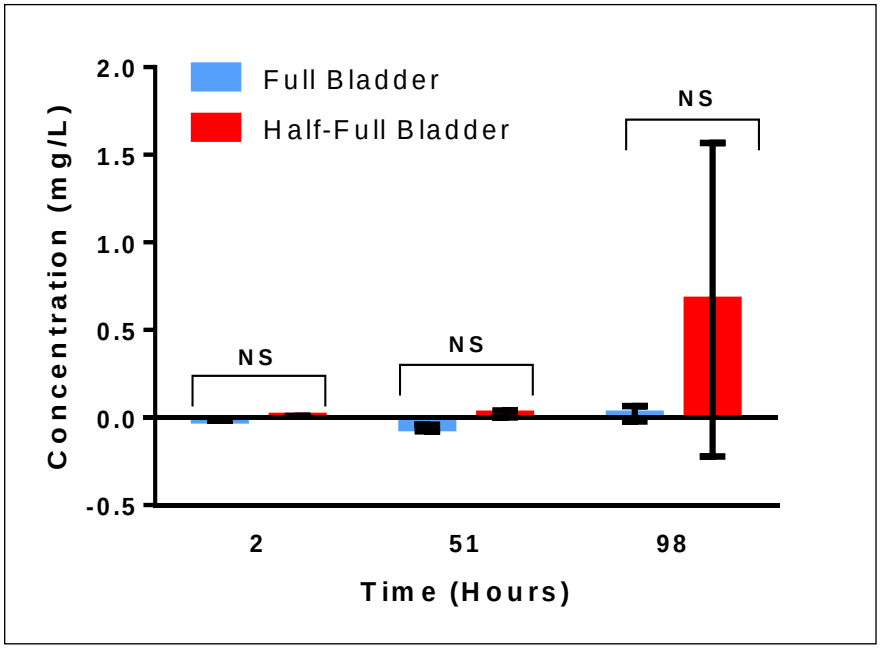

Figure 5.15 Two-Way ANOVA results of rhodamine B concentration in relation to the comparison of bladder volume (full and half-filled) of 100 mg/L rhodamine B in six fresh whole bladders at pH 5 over approximately 100 hrs (Error bars are ± S.D.)

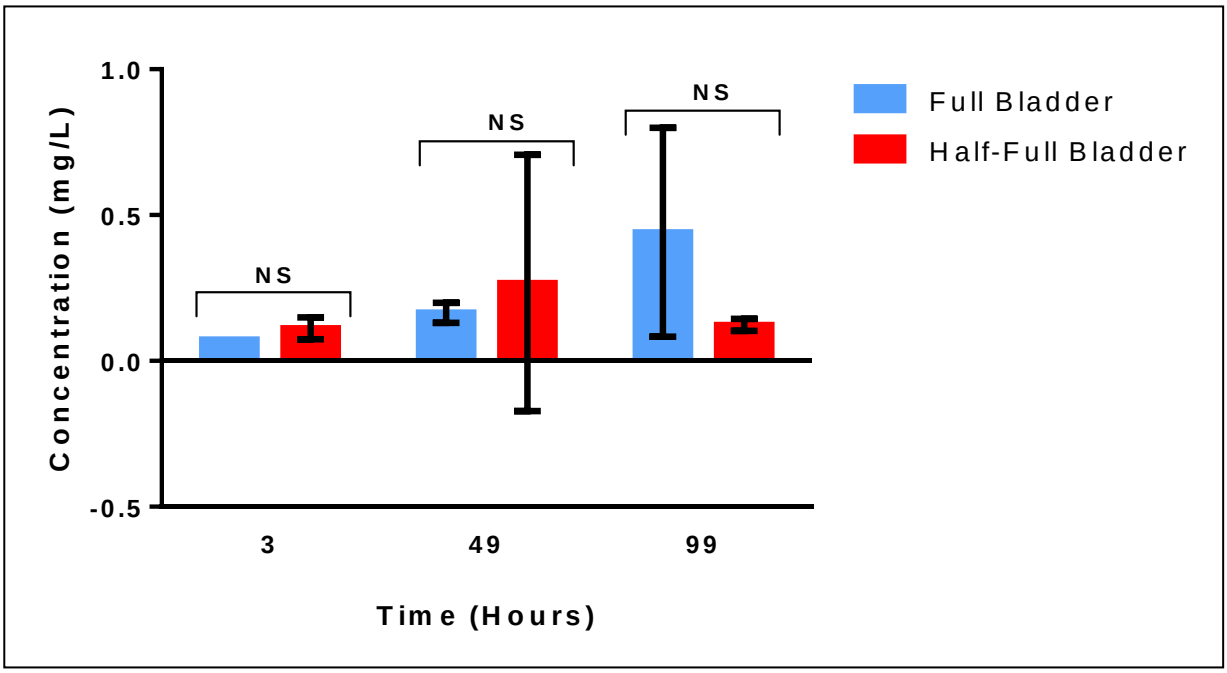

Figure 5.16 Two-Way ANOVA results of rhodamine B concentration in relation to the comparison of bladder volume (full and half-filled) of 200 mg/L rhodamine B in six fresh whole bladders at pH 5 over approximately 100 hrs (Error bars are ± S.D.)

5.3.3 Influence of pH on the amount of rhodamine B diffusing through whole bladders

The investigation into the effect of the solution pH on the cumulative concentrations of rhodamine B diffusing through whole bladders used two separate solution pH (pH 7.4 and pH 5) and included full bladders and half-full bladders. In addition, the other parameter included were two initial rhodamine B concentrations of 100 mg/L and 200 mg/L. A fresh set of calibration standards was prepared each day for each bladder experiment using the method from section 2.1.5.1 using PBS pH 7.4 and pH 5.

The comparison graphs show at pH 7.4 with a full bladder containing 100 mg/L rhodamine B has the highest cumulative concentration of rhodamine B, with a peak concentration of 3.5 ± 1.02 mg/L at 97 ± 0.03 hrs (See Figure 5.17). The other three sets of results show limited diffusion of the compound independent of the pH (See Figures 5.18 – 5.20). The peak concentration from these results was 1.24 ± 0.26 mg/L at 96 ± 0.01 hrs using 200 mg/L rhodamine B in full bladders at pH 7.4 (See Figure 5.19). The lowest cumulative concentration of rhodamine B was observed in pH 5 buffer solution with a full bladder and rhodamine B at a concentration of 100 mg/L, with the peak cumulative concentration < the 0.156 mg/L limit of detection (See Figure 5.17).

There was no significant difference between the cumulative rhodamine B concentrations in relation to half-filled bladders using 100 mg/L rhodamine B solution throughout the duration of the experiment (See Figure 5.22). The peak cumulative concentrations of rhodamine B were 0.96 ± 0.73 mg/L (pH 7.4) at 73 ± 0.01 hrs and 0.67 ± 0.89 mg/L (pH 5) at 97 ± 0.03 hrs (See Figure 5.18). There was significant difference ($p < 0.0001$) with three of the four sets of results at the later stages of the experiment at approximately 97 hrs post-mortem. The half-filled bladders at 200 mg/L had peak concentrations of 0.91 ± 0.22 mg/L at 97 ± 0.01 hrs (pH 7.4) and 0.3 ± 0.11 mg/L at 25 ± 0.01 hrs (pH 5) (See Figure 5.20). The full bladders at 100 mg/L had peak concentrations at 3.5 ± 1.02 mg/L at 97 ± 0.03 hrs (pH 7.4) and a concentration below the limit of detection for pH 5 (See Figure 5.17). Finally, the full bladders at 200 mg/L had peak concentrations of 1.24 ± 0.26 mg/L at 96 ± 0.02 hrs (pH 7.4) and 0.58 ± 0.95 mg/L at 73 ± 0.01 hrs (pH 5) (See Figure 5.19).

5.3.3.1 Bladder volume comparison of rhodamine B for whole bladders

The two bladder volumes used showed significant diffusion ($p < 0.0001$) for both the full volumes (100 mg/L and 200 mg/L rhodamine B) and one half-filled bladder (200 mg/L rhodamine B) experiment. The higher rhodamine B concentrations were observed at pH 7.4 during the last sampling in the experiment, at approximately 97 hrs post-mortem (See Figures 5.21, 5.23 and 5.24). The peak concentrations for the full bladders were 3.5 ± 1.02 mg/L at 97 ± 0.03 hrs (pH 7.4) at 100 mg/L and 1.24 ± 0.26 mg/L at 96 ± 0.02 hrs in pH 7.4 at 200 mg/L (See Figures 5.17 and 5.19). The peak concentrations using the half-filled bladders were 0.96 ± 0.73 mg/L (pH 7.4) at 73 ± 0.01 hrs at 100 mg/L and 0.91 ± 0.22 mg/L at 97 ± 0.01 hrs (pH 7.4) at 200 mg/L (See Figures 5.18 and 5.20).

5.3.3.2 Concentration comparison of rhodamine B for whole bladders

Comparing the two rhodamine B concentrations resulted in higher concentrations at 100 mg/L (pH 7.4) than 200 mg/L. The peak cumulative rhodamine B concentrations were 3.5 ± 1.02 mg/L at 97 ± 0.03 hrs for 100 mg/L (See Figure 5.17) and 1.24 ± 0.26 mg/L at 96 ± 0.01 hrs (See Figure 5.19) for 200 mg/L both in full bladders. Higher results were found at 100 mg/L (pH 5) in comparison with 200 mg/L however, the concentrations were lower than (pH 7.4). The peak cumulative rhodamine B concentrations were 0.67 ± 0.89 mg/L at 97 ± 0.03 hrs (See Figure 5.18) for 100 mg/L using half-filled bladders and 0.58 ± 0.36 mg/L at 73 ± 0.01 hrs for 200 mg/L (See Figure 5.19) in full bladders.

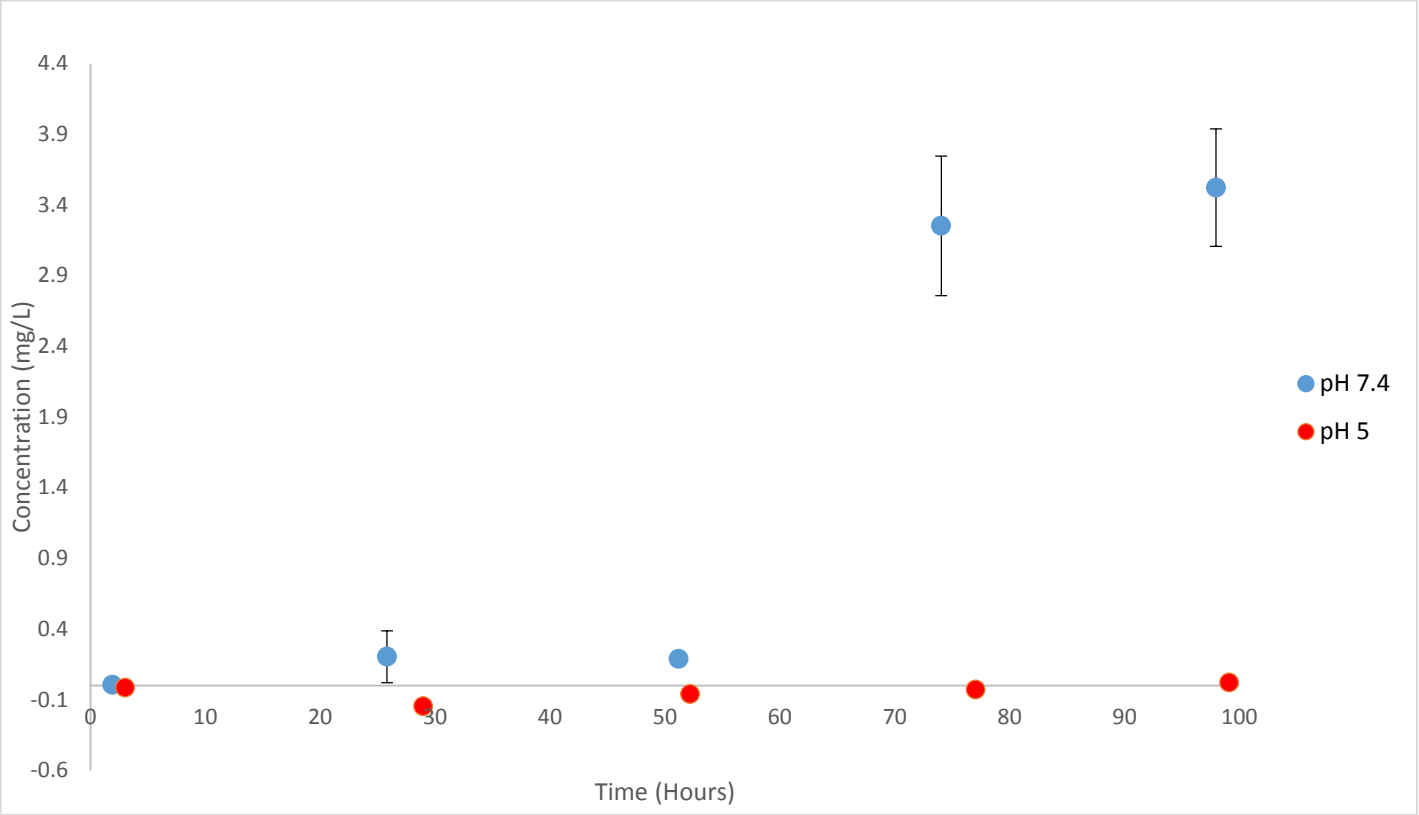

Figure 5.17 Average rhodamine B cumulative concentration six fresh whole bladders, comparison using 100 mg/L rhodamine B in six full bladders at pH 7.4 and pH 5 (n=6) (Error bars are ± S.D.)

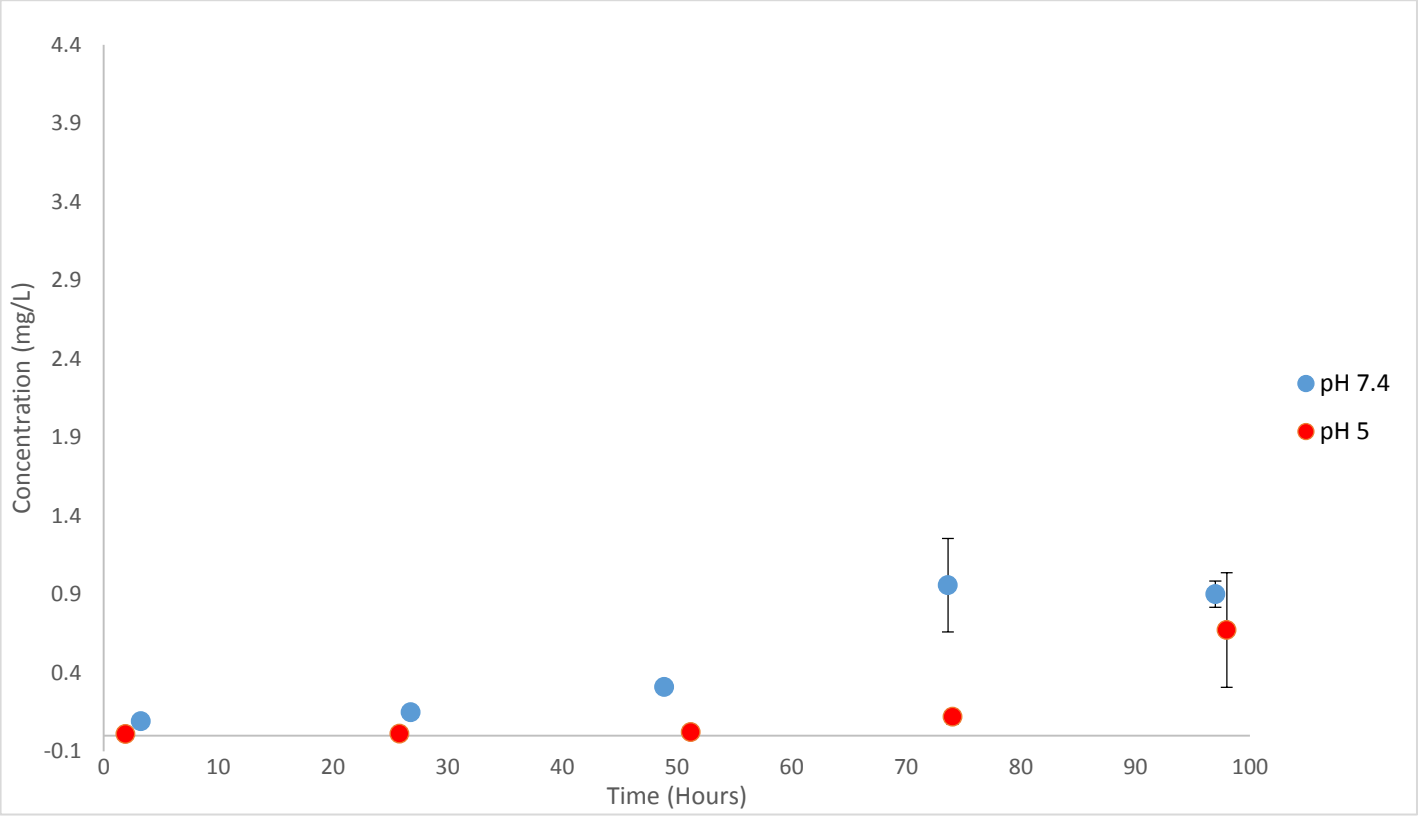

Figure 5.18 Average rhodamine B cumulative concentration six fresh whole bladders, comparison using 100 mg/L rhodamine B in six half-filled bladders at pH 7.4 and pH 5 (n=6) (Error bars are ± S.D.)

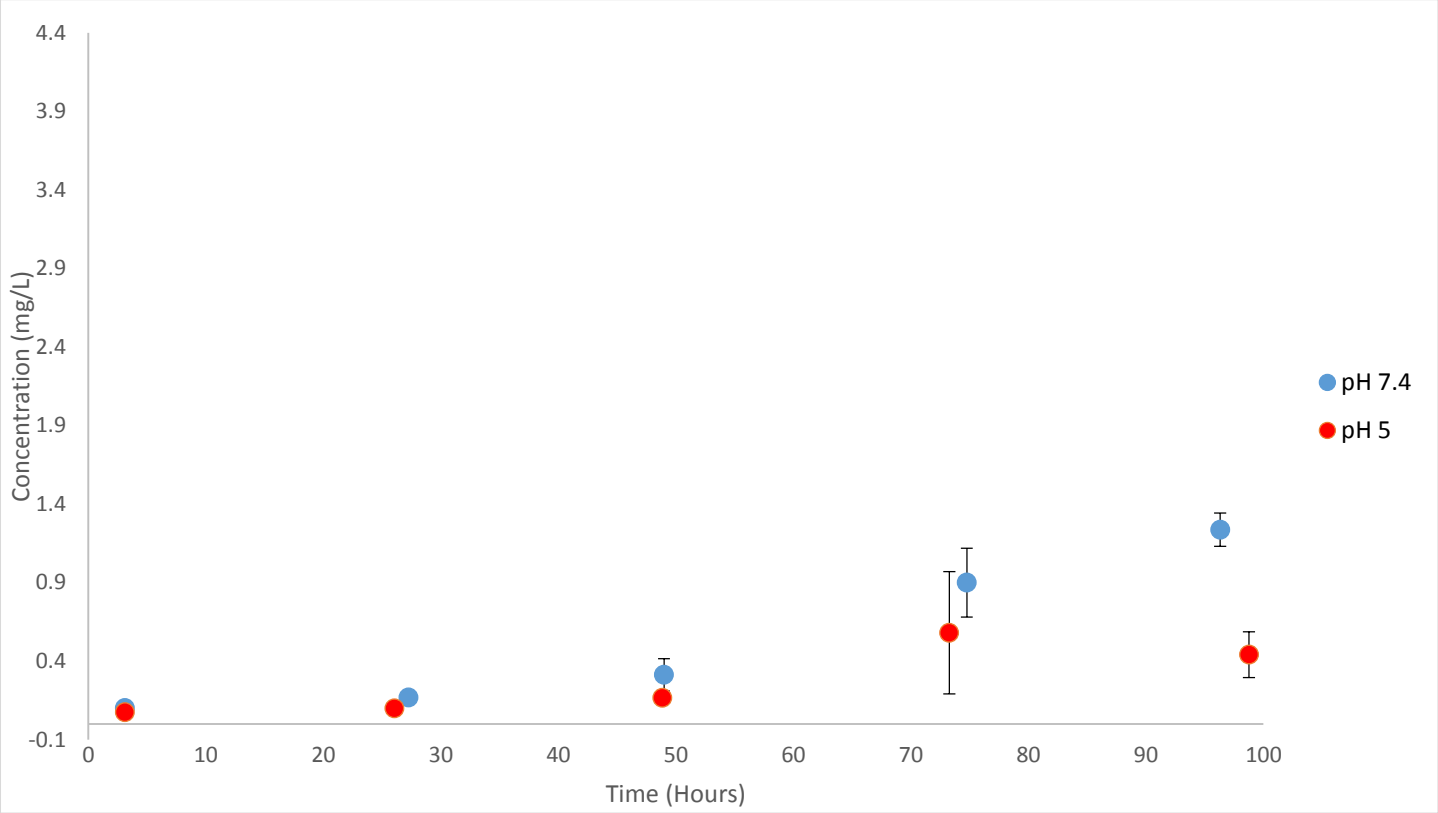

Figure 5.19 Average rhodamine B cumulative concentration six fresh whole bladders, comparison using 200 mg/L rhodamine B in six full bladders at pH 7.4 and pH 5 (n=6) (Error bars are ± S.D.)

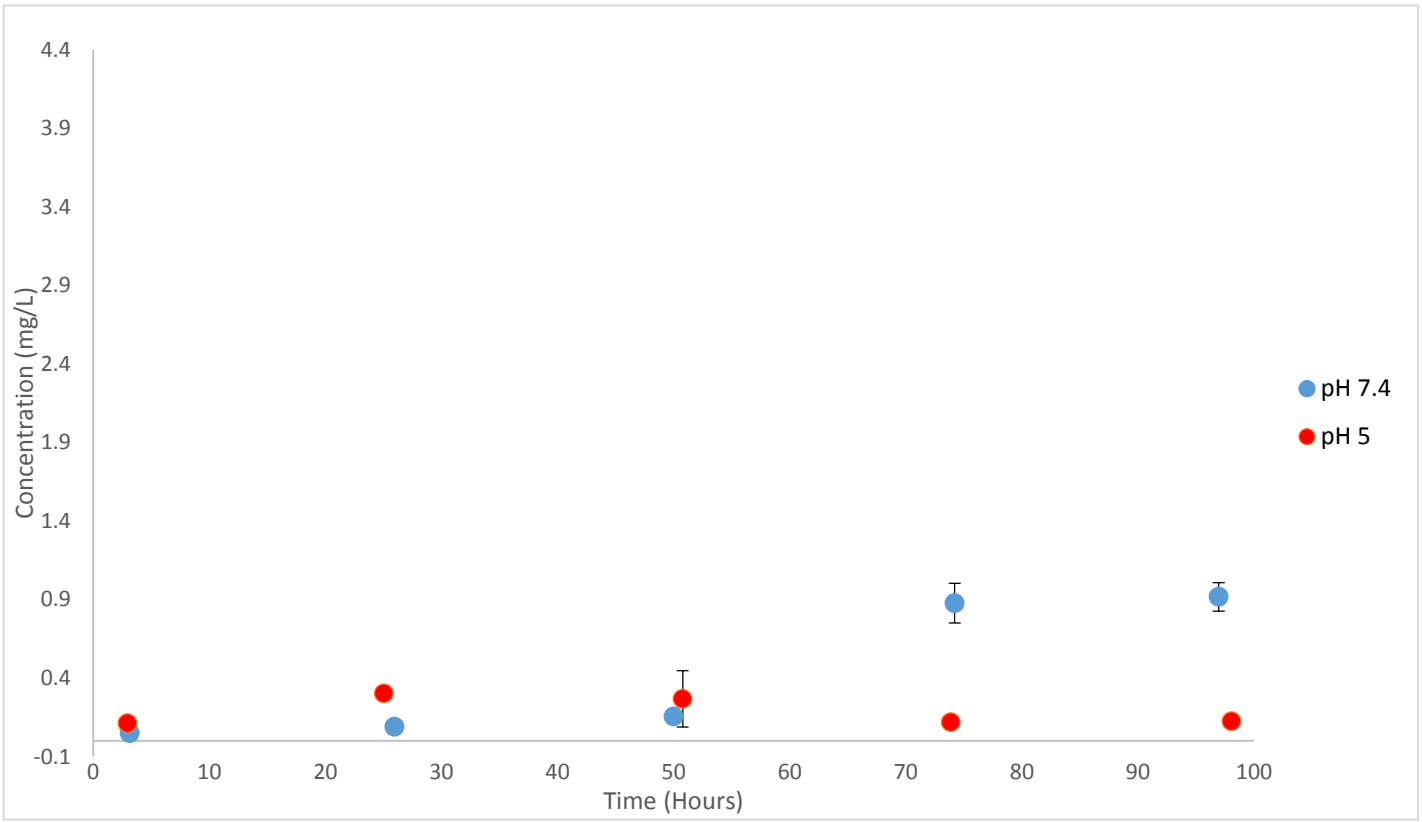

Figure 5.20 Average rhodamine B cumulative concentration six fresh whole bladders, comparison using 200 mg/L rhodamine B in six half-filled bladders at pH 7.4 and pH 5 (n=6) (Error bars are ± S.D.)

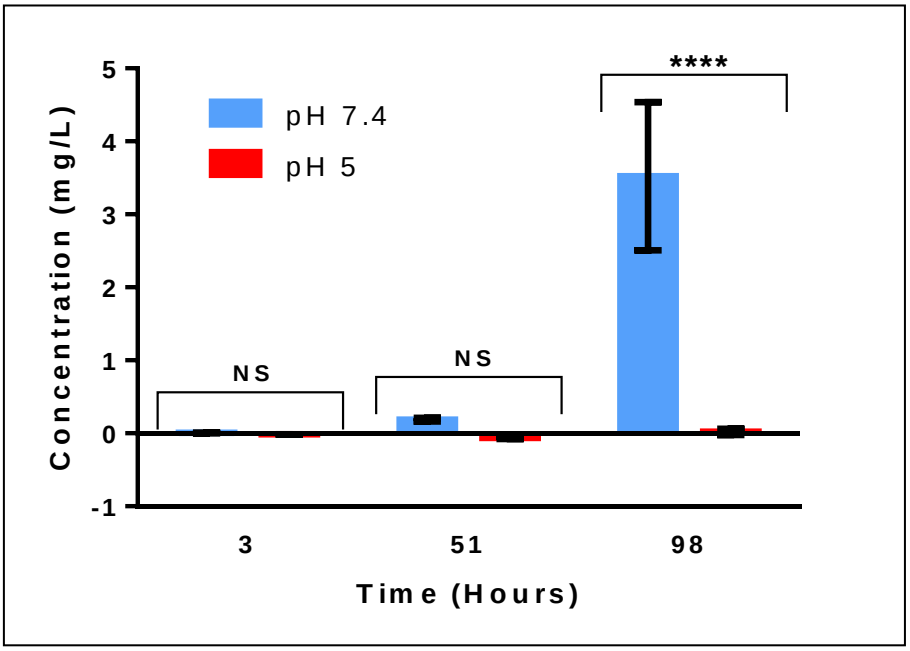

Figure 5.21 Two-Way ANOVA results of rhodamine B concentration in relation to the comparison of two solution pH values (pH 7.4 and pH 5) that submerged six fresh whole bladders at full volume individually, containing 100 mg/L rhodamine B over approximately 100 hrs (Error bars are ± S.D.)

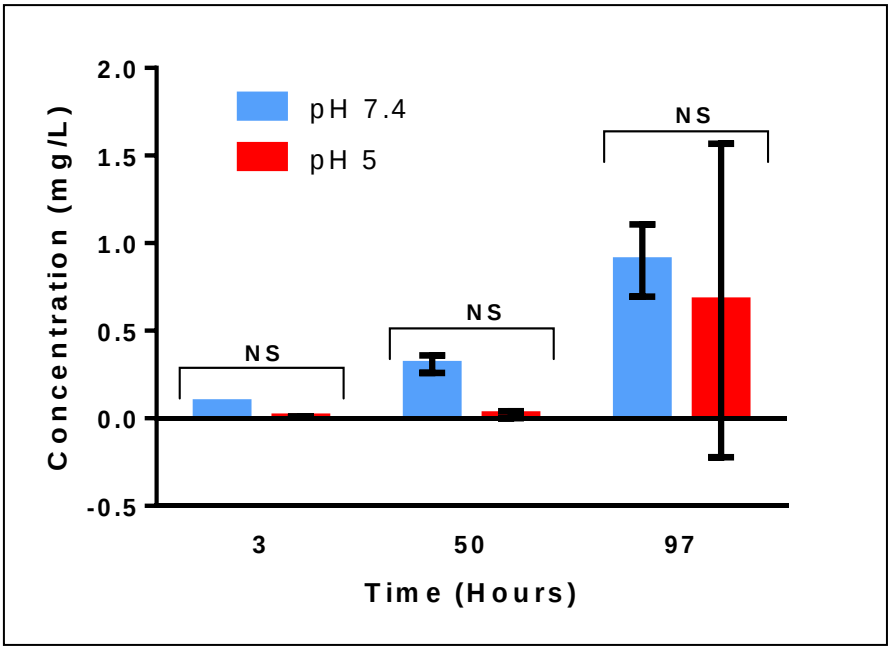

Figure 5.22 Two-Way ANOVA results of rhodamine B concentration in relation to the comparison of two solution pH values (pH 7.4 and pH 5) that submerged six fresh whole bladders at half-full volume individually, containing 100 mg/L rhodamine B over approximately 100 hrs (Error bars are ± S.D.)

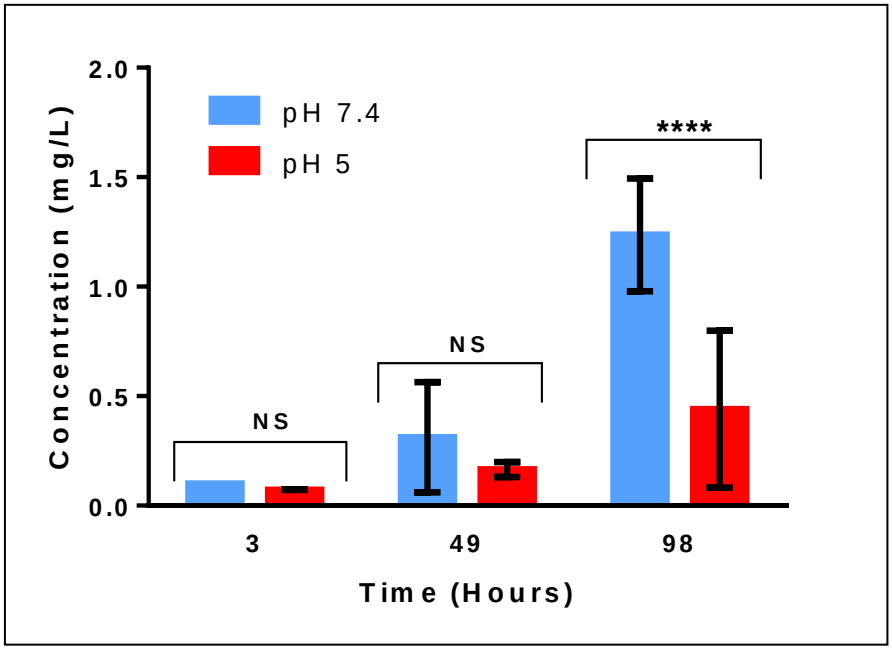

Figure 5.23 Two-Way ANOVA results of rhodamine B concentration in relation to the comparison of two solution pH values (pH 7.4 and pH 5) that submerged six fresh whole bladders at full volume individually, containing 200 mg/L rhodamine B over approximately 100 hrs (Error bars are ± S.D.)

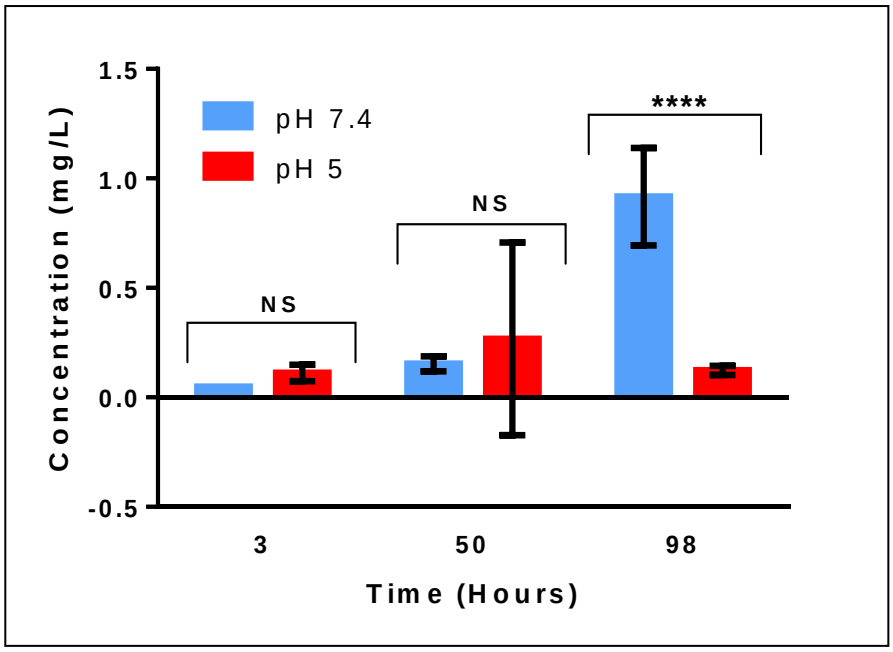

Figure 5.24 Two-Way ANOVA results of rhodamine B concentration in relation to the comparison of two solution pH values (pH 7.4 and pH 5) that submerged six fresh whole bladders at half-full volume individually, containing 200 mg/L rhodamine B over approximately 100 hrs (Error bars are ± S.D.)

5.4 Discussion

These experiments have shown that concentration does not affect diffusion of rhodamine B as much as volume through bladder tissue within the first 100 hrs post-mortem. This is due to the initial 100 mg/L rhodamine B solution resulting in higher cumulative rhodamine B concentration in comparison with 200 mg/L rhodamine B. The peak concentrations were 3.5 ± 1.02 mg/L at 100 mg/L rhodamine B and 1.23 ± 0.26 mg/L at 200 mg/L rhodamine B in pH 7.4. In addition, the bladder volume shows significant difference with higher rhodamine B cumulative concentrations with full bladders (3.5 ± 1.02 mg/L) in comparison with 0.95 ± 0.73 mg/L using half-filled bladders at pH 7.4. However, the results are affected by pH as the differences only occur at pH 7.4. The solution pH shows a possibility of having an effect on the cumulative concentrations of rhodamine B during the later stages of the initial 100 hours post-mortem due to the significant differences (p less than 0.0001) for rhodamine B diffusing through the tissue. The results from the full bladders using 200 mg/L of rhodamine B resulted in peak cumulative concentrations of rhodamine B at 1.24 ± 0.26 mg/L in pH 7.4 and 0.58 ± 0.95 mg/L in pH 5. The bladders used in the experiment were in different states including contracted bladders with no urine present and distended bladders with urine present, which could affect bladder thickness. This leads to a limitation of this type of experiment as the thickness of the bladders could not be determined. This was due to the bladders being kept intact; therefore, the digital callipers used to measure tissue thickness could not be used. However, as shown in chapter 4 the thickness of the bladder was not shown to have noticeable influence on the diffusion in this model so is unlikely to have much of an influence on diffusion in the intact bladder model. However, this would mimic the conditions found in deceased individuals and therefore this is good model.

The lower concentration of rhodamine B diffusing from the bladder at the higher pH shows that a larger amount of drug does not need to be present to increase the concentration. However, at the lower pH this shows the opposite where the larger amount of drug was detected at a later stage. This could be mainly due to bladder degradation as this is at a late stage in the experiment, which was approximately 97 hrs after death. The bladder could have degraded during the experiment producing an opening however, the bladders were not specifically checked for possible openings. This would be a factor to include in any future work. The larger concentration of rhodamine B, even with the dilution from the outside solution, would have a larger spike in concentration than the lower concentration. As a result, this factor would not be the main effect on the diffusion rate of rhodamine B.

In addition, the PMI is within the time recorded of the individual found with increased drug concentrations in the femoral vein (Moriya and Hashimoto, 2001). However, it took 97 hrs (~ 4 days) for the drug concentration to reach 3.5 ± 1.02 mg/L outside the bladder and including diffusion and dilution that would occur between the bladder and the femoral vein any changes in the femoral vein would be small. In humans, there has been shown to be some peritoneal cavity fluid present in both males (3.0 ± 2.7 ml) and postmenopausal women (2.3 ± 2.0 ml) (Yoshikawa *et al.*, 2013). Dilution of drugs diffusing out from the bladder would occur at this stage with an alteration dependant on the peritoneal fluid values. There would be an increase in rhodamine B concentration (assuming the initial concentration of rhodamine B was in the volume of the donor chamber in the Franz cell - 5ml) in males with 5.83 ± 37.37 mg/L and females with 7.61 ± 36.07 mg/L. This rhodamine B

concentration would then be diluted during the diffusion from the peritoneal cavity fluid to the surrounding area including the femoral vein. These values are under the assumption of healthy individuals however, in some cases certain diseases including peritonitis causes ascites, which is the accumulation of peritoneal fluid and therefore this would dilute any drugs with the increase of fluid outside of the bladder (Staff, 2016).

The significant difference between the bladder volumes at only one pH (pH 7.4) and at the late stage of the experiment show this is not a main factor that could affect the diffusion rate of the rhodamine B. The significant increase in diffusion at the end of the experiment could relate more to possible tissue degradation than to volume. After approximately four days, the tissue could have degraded to a point where an opening was created allowing for movement of rhodamine B into the acceptor chamber. The full bladders at pH 7.4 showed higher rhodamine B concentrations than at half-filled bladders using 100 mg/L rhodamine B ($p < 0.0001$) at 97 ± 0.03 hrs (See Figure 5.9). As a result, this could not be a main factor that would affect drug diffusion from whole bladders.

Three sets of results showed consistently higher diffusion for rhodamine B at pH 7.4. The pKa of rhodamine B is 4.2 (Zhang *et al.*, 2011) resulting in a 99.9% ionised compound at the higher pH, this is consistent with the study showing ionised compounds preferentially diffuse through bladder tissue compared to unionised compounds (Moch, Salmon and Armesto, 2014). However, all the results showing significantly higher diffusion for pH 7.4 are at the end of the experiment, this could suggest that the bladder degradation is the main driving force behind the drug release due to tissue breakdown. However, as pH 7.4 is significantly different from pH 5 in all cases this could still include the possibility that even with decomposition the pH still has an effect on the amount of diffusion occurring regardless of concentration and volume.

Even with one factor possibly affecting the amount of rhodamine B diffusing through whole bladders, the peak cumulative concentration of rhodamine B over all the experiments was 3.5 ± 1.02 mg/L from initial concentrations of 100 mg/L and 200 mg/L within the bladder. This reduces the possibility of large concentrations leaking from the bladder and diffusing to the femoral vein from this model. In addition, the largest cumulative concentration resulted from physiological pH and the body would not be stabilised at this pH long after death it would reduce, which showed a reduction in rhodamine B concentration at pH 5. This could be due to rhodamine B possibly having a higher affinity for the bladder tissue as it has a log P of 2.43, showing it has higher affinity for the organic partition, which was determined in chapter 4 section 4.3.4.1. This experiment only used intact bladders; this study would not cover damaged bladders including openings in the bladders from trauma. In addition, results at pH 7.4 showing more diffusion would not relate to casework as after death the pH within the urine has been shown to decrease to pH 4.6 (Cook, Strauss and Caplan, 2007) so would be observing the type of diffusion shown by the pH 5 results. The later stage of decomposition is classed as putrefaction, which does increase the pH within the body however not to the extent of physiological pH. This shows that there would be very limited diffusion from whole bladders post-mortem.

Overall, the experiments showed that significant differences of rhodamine B cumulative concentrations with the highest concentration at 3.5 ± 1.02 mg/L only occurred after approximately 97 hrs post-mortem. Therefore, no large drug concentrations would be released from the bladder within the first 5 days after death. Any drug release would diffuse into the surrounding peritoneal cavity, which could cause an increase in drug concentrations in surrounding sampling sites. However, due to the small amounts of drugs recorded during this experiment this would probably not affect the interpretation of the drug concentrations in the other sampling sites including the femoral vein. This is corroborated by the length of time the largest rhodamine B drug concentration was recorded, 97 hrs post-mortem, and then the diffusion and dilution that the drug would undergo to reach to femoral vein may not be possible within the 9 days suggested (Moriya and Hashimoto, 2001). In addition, upon reaching the femoral vein the drug would have to diffuse through the wall of the vein, which has been recorded as an efflux to take between 2-20 hrs with a linear increase in drug diffusion up to 80 hrs post-mortem (Skopp *et al.*, 1997).

Rhodamine B has resulted in 3.5 ± 1.02 mg/L being able to diffuse through the bladder 97 hrs after death, this drug would then increase in concentration in the peritoneal fluid to approximately 6.72 ± 36.72 (average of male and female peritoneal fluid rhodamine B concentrations). There would be an undetermined time to diffuse between the bladder and the femoral vein however, once at the femoral vein there is a range of 2-20 hours for the drug to diffuse into the femoral vein and up to a further 80 hours to increase the concentration within the vein. The average sample volume of the femoral vein is 10 ml (Cooper, Paterson and Osselton, 2010), the average rhodamine B concentration within the femoral vein would be 1.75 ± 1.02 mg/L. The overall time for this process, between death and the drug altering in concentration in the femoral vein, to take place would be at least between 179 – 197 hrs post-mortem. *In vivo* work would allow for the determination of the direction of a drug that could be released from the bladder post-mortem and observe if the drug could reach the femoral vein within 9 days after death.

Chapter 6 – Study of the Diffusion of Drugs from the Whole Rat Bladder using X-Ray Micro-CT

6.1 Introduction

The previous work in this thesis has focussed solely on the bladder in isolated conditions. It is however important to study as closely as possible how the bladder would mimic real casework. In relation to *in vivo* work the bladder would be within the body cavity after death and degradation could be altered as surrounded by other tissues and fluids in the peritoneal cavity. As a result, rats were used in this experiment where the bladder was kept intact inside the rat and the bladder was catheterised. A computed tomography (CT) contrast agent was inserted, sealing the bladder and scanning the rat on a micro-CT over nine days. The significance of the duration of the experiment matched the length of time in a real case in Japan where it was suggested that drug diffusion from the bladder had increased the drug concentrations (dihydrocodeine and diphenhydramine) in the femoral vein to those above that at the time of death (Moriya and Hashimoto, 2001).

Previously work has been carried out using X-Ray images determining the effect of molecular weight and lipophilicity on the ease of movement of drugs around the body after death, showing that the smaller, aminohippuric acid, and the more lipophilic, Lipiodol ultra fluid, compounds were mechanically moved around the body mainly by muscle contraction (Zapata, Luna, 1989). Movement around the heart into adjacent vessels occurred within the first hour after death and this movement continued until the conclusion of the experiment at 72 hrs post-mortem. This shows that after death drugs can move around the body due to blood movement which could alter drug concentrations at specific sites post-mortem (Zapata, Luna, 1989). Drugs could also be distributed mechanically around the body due to the movement of the body after death in particular, transport to the mortuary (Fallani, 1961; Anderson and Jones, 1990), it is also likely that any mechanical ventilation or cardiopulmonary resuscitation (CPR) would also cause movement of the blood (and thus redistribution of drugs after death (Yonemitsu, 1991)).

This research has been continued using CT and contrast media to observe changes in tissues over time. Post-mortem computed tomography (PMCT) has been developed as another tool in death investigations and used for a number of reasons including changes in heart muscle thickness after death (Okuma *et al.*, 2013). In addition, whole-body PMCT has been used to identify markers associated with overdose related deaths, specifically opioids including heroin and methadone. The results have suggested brain and lung oedema and a full urinary bladder are indications of an opioid overdose related death (Winklhofer, Surer and Ampanozi, 2014). Another study involving PMCT inferred that distended bladders suggested intoxication; this study covered a wide range of drugs including multiple drugs and alcohol. The mean bladder volume that was marked as the volume where intoxication should be considered was 330 ml as this volume was not present in non-intoxicated individuals (Rohner and Franckenberg, 2013). PMCT has been suggested as a replacement for post-mortem examinations due to the non-invasive nature of the procedure, which would be preferred by the public due to the invasive nature of the autopsy process. There are limitations as certain causes of death could not be identified using CT alone including pneumonia and ischemic heart disease (Roberts *et al.*, 2012). PMCT would be less invasive however, if it were to replace the traditional autopsy it would need to combine with a number of other areas for a comprehensive investigation and these areas would include histology, toxicology and case history review (Saunders *et al.*, 2011). These studies show that CT is a valuable resource that can be used in a range of ways to determine post-mortem changes within the body.

In order to visualise post-mortem changes, especially with drug movement, compounds must be used that can be visible on an X-Ray-CT image. CT images are produced by materials of various densities absorbing X-rays to different extents, the denser the material the brighter the area on the CT image (Hrvoje, Lusic, 2014). The ideal contrast agent would be similar in properties to the drugs being investigated, visible on the CT image and water-soluble. Contrast media are usually selected due to their non-toxic properties however; in this case, as this research is post-mortem based, toxicity to the subjects was not an issue. The usual element required for contrast media is iodine or barium, however barium is sparingly soluble in water creating a suspension, which would not be suitable for this research (Bosniak, 1980). Iodine-based contrast media has been favoured for CT imaging and used in research to highlight brain tissue (de Crespigny A, 2008), however medical grade contrast media have short half-lives ranging between 1-2 hrs with all the contrast media being eliminated within 24 hrs (Geenen, Kingma and van der Molen, 2013) as medical procedures are short-term. As post-mortem changes can take place over longer time periods (hrs to days) it is important to have a contrast agent that will be visible for that length of time. As iodine is a good contrast agent with an atomic number of 53, elements with a similar atomic number and longer half-life could replace iodine. Silver, with the atomic number of 47, has been used as a contrast agent in rats investigating the clearance rate over a four month period showing the sites still contain the nanoparticles after this period giving a half-life range, dependant on the biological tissue, between 1-4 months (Lee and Kim, 2013). This half-life is more suitable for long-term studies. More specifically, silver was investigated on a Micro-CT and was identified throughout the seven day experiment at a low concentration of 37 mM (Zou *et al.*, 2015). This demonstrates silver can be a suitable alternative to iodine with a longer half-life and only low concentrations needed to be identified on Micro-CT.

The previous research carried out on Micro-CT show that it is possible to determine the movement of a CT-visible drug from the bladder post-mortem. In addition, due to the ethical restrictions with using human cadavers, the use of rats for this research could be beneficial due to the small size, the ability to reproduce the conditions due to availability of the animals and can be comparable to human bladders (Hicks, 1977). Specifically, silver nitrate has been used in numerous CT-based studies including tooth development in mice (Raj *et al.*, 2014) and human lungs (Watz, Breithecker, Rau, 2005).

6.2 Aims

The aim of this chapter of the thesis was to determine if a rat CT model can be used to measure and visualise post-mortem diffusion from the bladder.

6.3 Results

A urinary catheter was used to insert 0.25 ml of 1M silver nitrate into the bladder of a rat to determine the diffusion of the sliver nitrate from the bladder, the length of the experimental time mimicked the case report of the individual who was found after nine days (Moriya and Hashimoto, 2001). This period would allow to determine if the drugs would have enough time reach and then to diffuse in the femoral vein, and to investigate any possible breakdown of the bladder tissue. As people can be found deceased at home,

previous work on alcohol diffusion from a cadaver model and work in this thesis on bladder tissue has shown that diffusion is slowed at lower (5°C) temperatures (Smith, 1995). This model of diffusion from the bladder was carried out at 20°C, which a body would equilibrate to in an average room. The average temperature recorded over the course of the experiment in the fume cupboard, the storage place for the rat, was 20.8 ± 0.96 °C.

The time of death of the rat could not be identified as the rat was purchased frozen, therefore this is a limitation of the experiment as could not observe changes from the nine days immediately following death. As a result, this experiment was observing the change in the bladder over nine days post-mortem after the rat had been thawed and catheterised with this point taken as time zero. The freezing and thawing of the tissues was not classed as a limitation as it was demonstrated earlier in this thesis, section 4.3.3, that freezing and thawing bladder tissue sections did not have an effect on diffusion.

Over the course of the experiment, a number of bladder changes were observed. Initially, the bladder was easily identified and showed an even surface resulting in a filled bladder, with no damage that would allow for leakage (See Figure 6.1 and 6.2). The bladder was filled using a syringe and catheter, therefore when the bladder was filled there was residual solution that filled the urethra and this can be seen in figure 6.2. The comparison images from the CT show that there are numerous air bubbles in the body cavity, which are shown by the darker sections (See Figure 6.3). CT works based on density, the higher the density the lighter the area looks on the CT image; this is the reason for the air bubbles to be shown as the darkest areas. The bladder is shown as a solid mass in these images for day one (Figure 6.3). The second day shows similar results to the first day with no change in the 3D rendered image with the bladder in a similar shape and position (See Figure 6.4). However, in figure 6.5 the individual CT images show a disruption in the centre of the bladder where there is an air bubble. This air bubble is present due to the displacement of the silver nitrate, which could have occurred due to a number of reasons including diffusion of the compound into bladder tissue or displacement due to movement of the rat between storage and the Micro-CT.

Day six shows the bladder has reduced in size (See Figure 6.6) and shifted further down in the peritoneal cavity (See Figure 6.7), which could be due to a number of factors including putrefactive gases causing pressure within the cavity or the change in position of the rat for analysis. The silver nitrate in the urethra is still visible however; there is a possibility of this compound leaking from the bladder on the right underside of the bladder shown by a mass (See Figure 6.7). The pressure caused by putrefactive gas could contribute to any solution leakage forcing the contrast agent out of the bladder through the possible hole in the bladder. There is a scale on the composition images for day six, however due to the angle of the images an estimation of the distance from the femur cannot be identified (See Figure 6.8). The composition image shows large air bubbles in the body cavity and the air bubble from day two is still present within the bladder. Sections of bone were highlighted in this image to show the similarity of the density of the silver nitrate to bone, this allows both to be isolated from the rest of the image including tissue and the plastic container (See Figure 6.8).

Day seven has similar results as the previous day however; the possible leakage of silver nitrate shows to be moving further down the peritoneal cavity, below the bladder (See Figure 6.9). There is a front view of the rat in the composition image on day seven (See Figure 6.10, Top Right image). The scale on the image can be

used and it shows the leaked silver nitrate is approximately 10 mm from the femur. The actual distance cannot be determined for the femoral vein as it cannot be currently identified (See section 3.3.5.4). The air bubble in the composition image shows to be increasing (See Figure 6.10). The top of the bladder seems to keep the shape on day eight, however the silver nitrate outside of the bladder cannot be seen in the image (See Figure 6.11 and 6.12). The air bubble can be easily identified in both the 3D rendered image (See Figure 6.11), and the composition images (See Figure 6.12). The final day of the experiment shows the bladder is still visible and the bladder shape is still intact even with the reduction in size and some misshaping (See Figure 6.13) with the air bubble visible (See Figure 6.14). The composition image shows the increase in the air bubble and the displacement of the bladder to a lower position within the peritoneal cavity, which could be due to putrefactive gases or the vertical position required for analysis (See Figure 6.15).

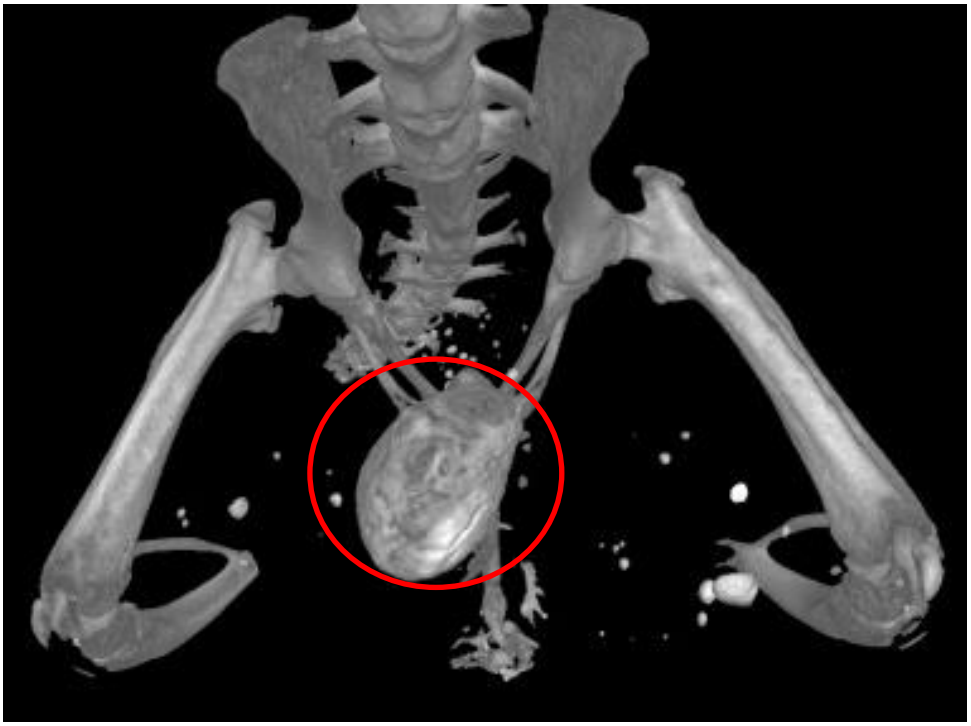

Figure 6.1 Day one, CT image of the top view of the rat skeleton and silver nitrate in the bladder (circled in red)

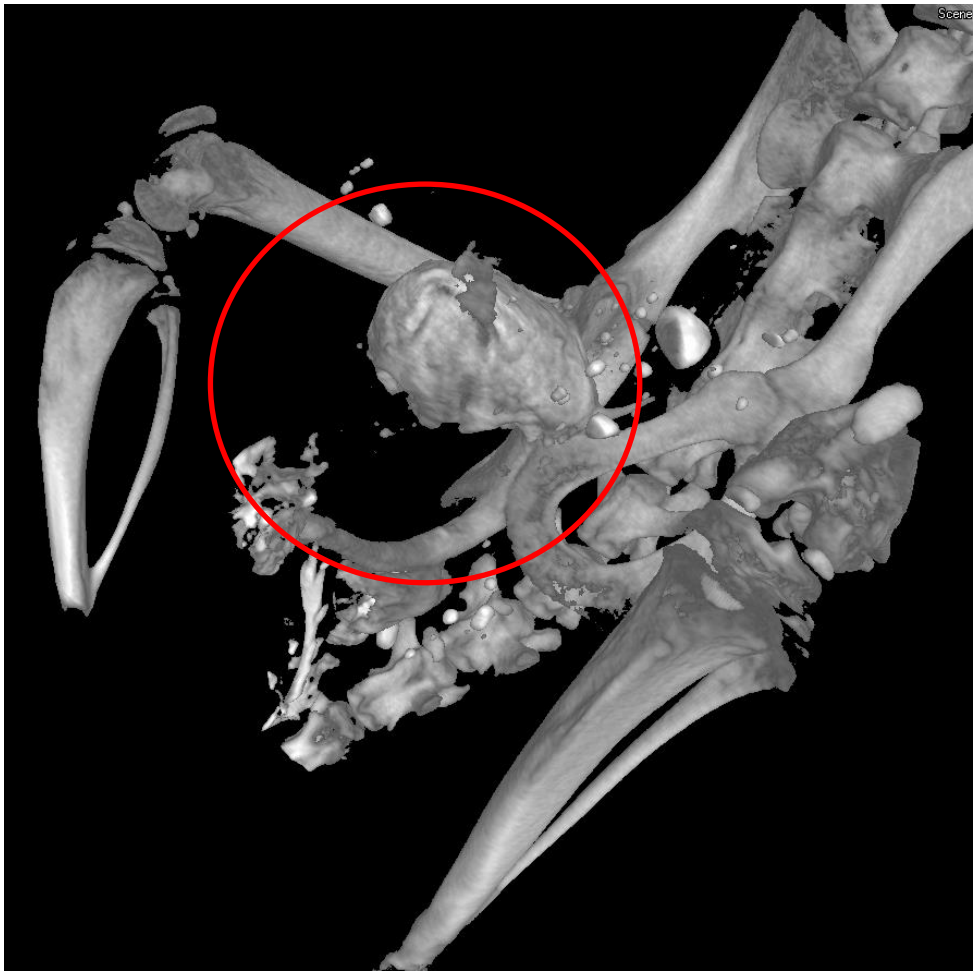

Figure 6.2 Day one, CT image of the angled side view of the rat skeleton and silver nitrate in the bladder and urethra (circled in red)

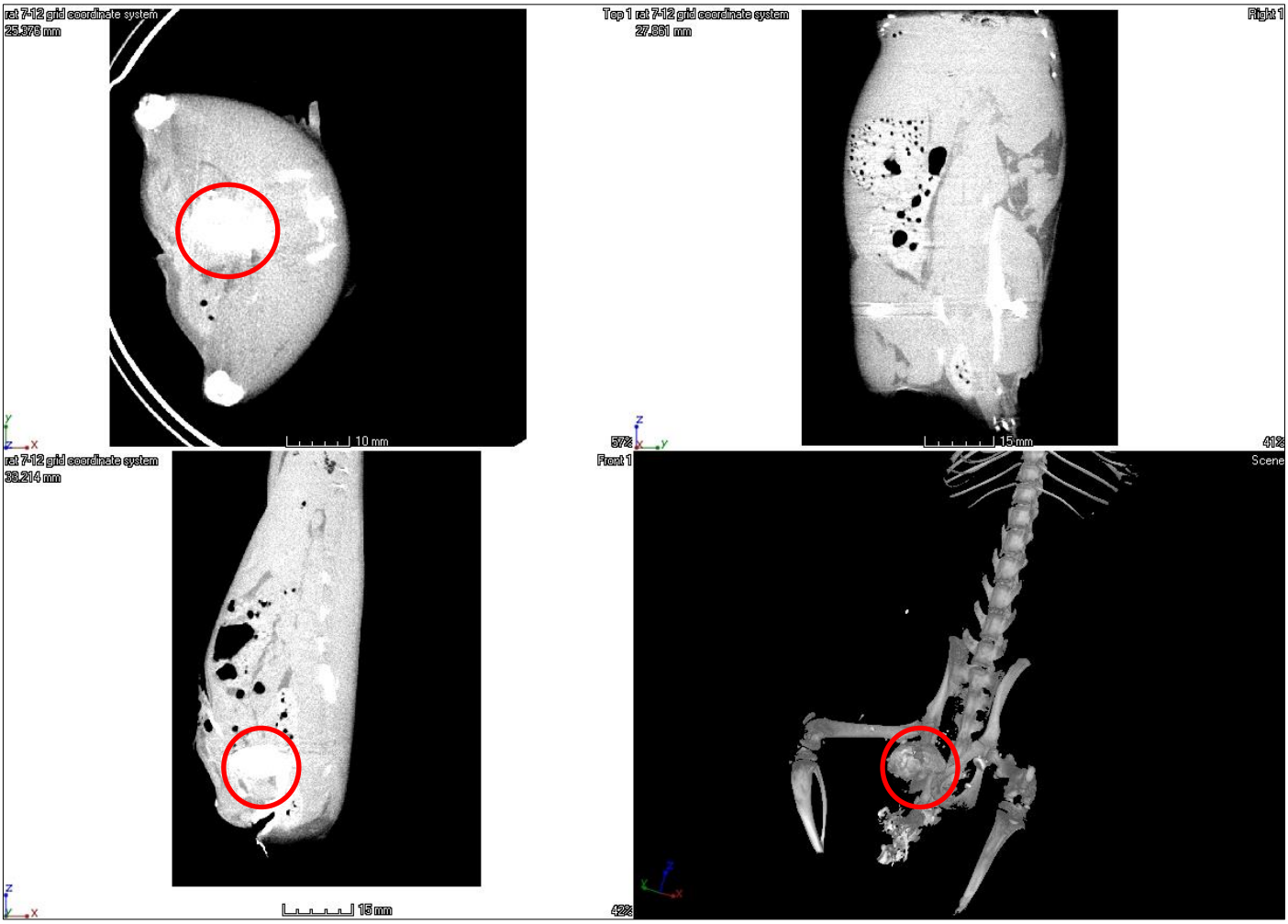

Figure 6.3 Day one, top view (top left image), front view (top right image), left side view (bottom left image) of the abdomen of the whole rat including tissue, the scaled back image of the skeleton (bottom right image) and the CT drug silver nitrate within the bladder (circled in red)

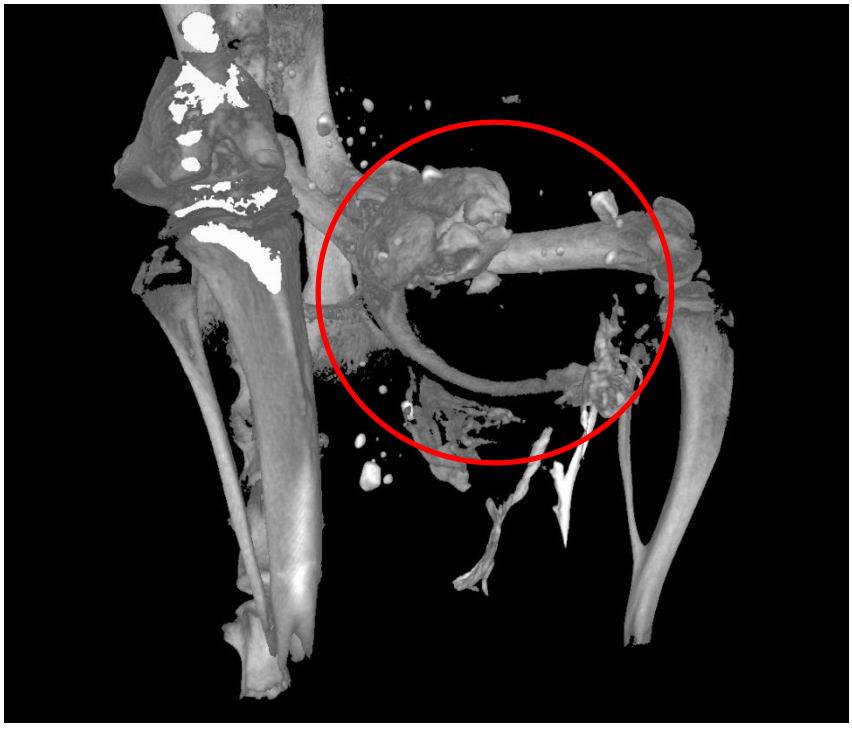

Figure 6.4 Day two, CT image of the angled side view of the rat skeleton and silver nitrate in the bladder and urethra (circled in red)

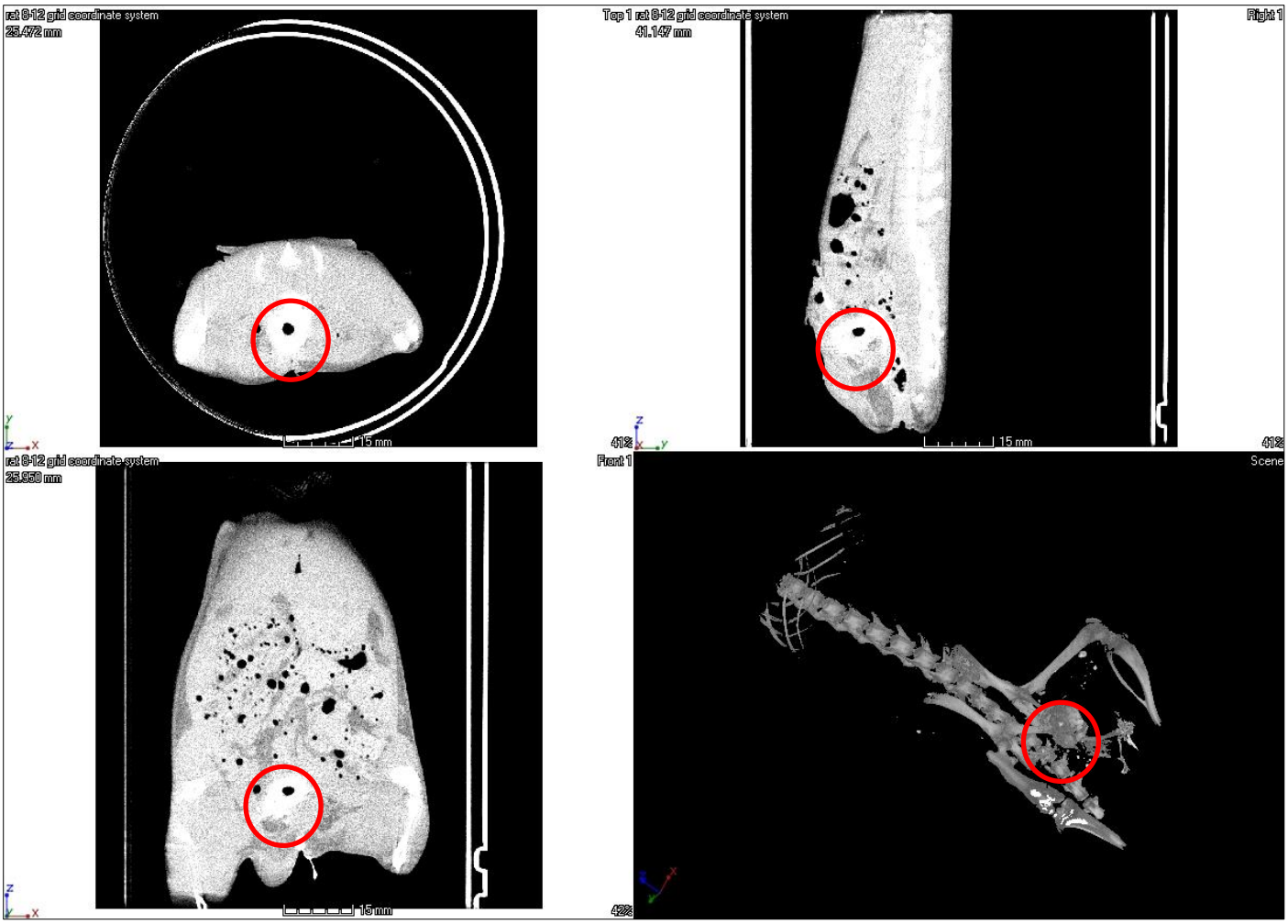

Figure 6.5 Day two, top view (top left image), left side view (top right image), front view (bottom left image) of the abdomen of the whole rat including tissue, the scaled back image of the skeleton (bottom right image) and the CT drug, silver nitrate, within the bladder (circled in red)

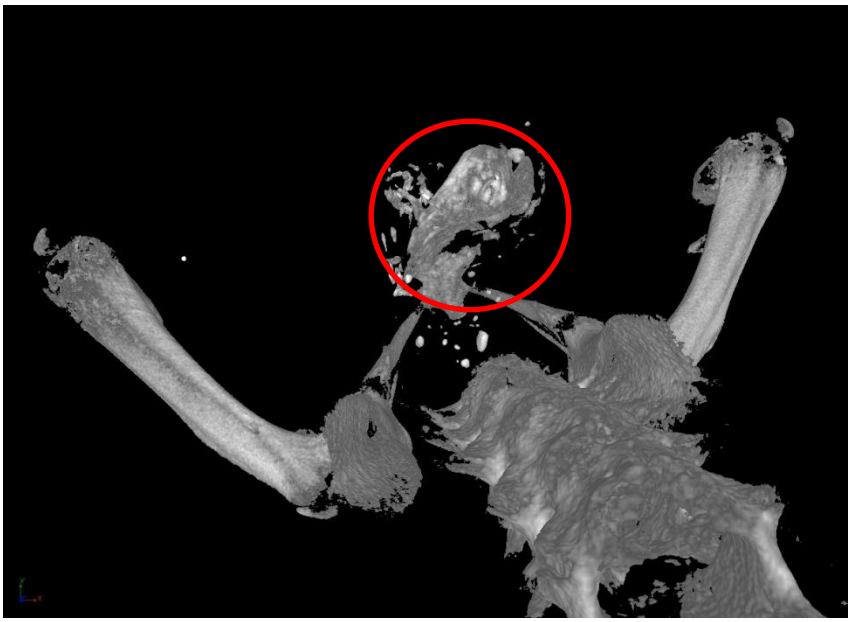

Figure 6.6 Day six, CT image of the top view of the rat skeleton and silver nitrate in the bladder (circled in red)

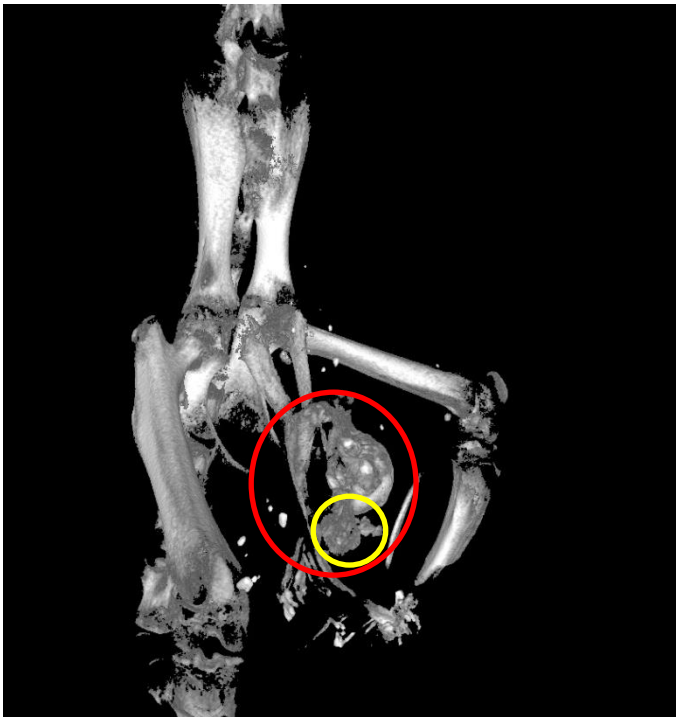

Figure 6.7 Day six, CT image of the angled side view of the rat skeleton and silver nitrate in the bladder and urethra (circled in red). Possible leakage of silver nitrate from the bladder (circled in yellow).

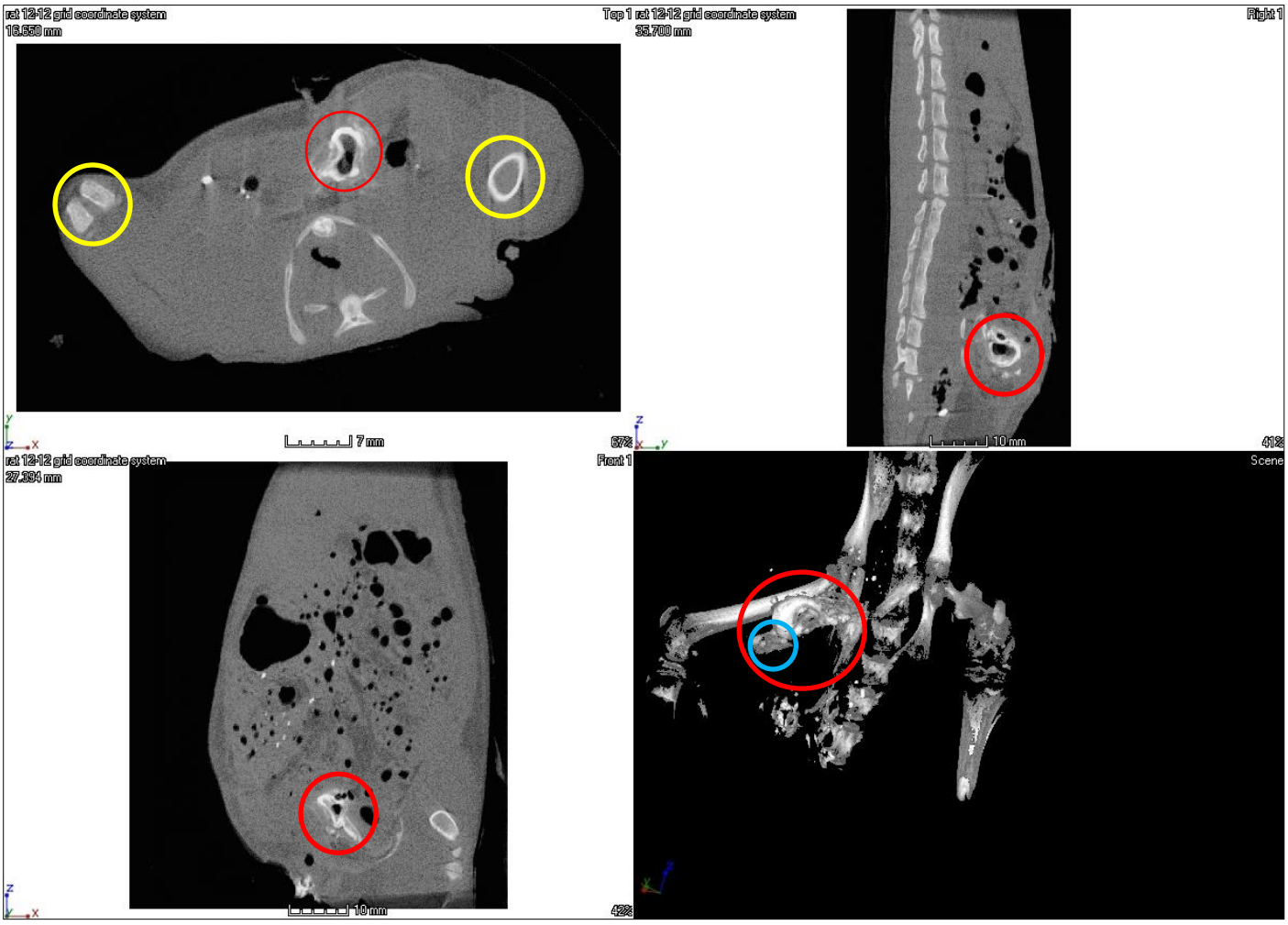

Figure 6.8 Day six, top view (top left image) with bones circled in yellow for density comparison. The right side view (top right image), front view (bottom left image) of the abdomen of the whole rat including tissue, the scaled back image of the skeleton (bottom right image) with the bladder and urethra (circled in red). Possible leaked silver nitrate (circled in blue).

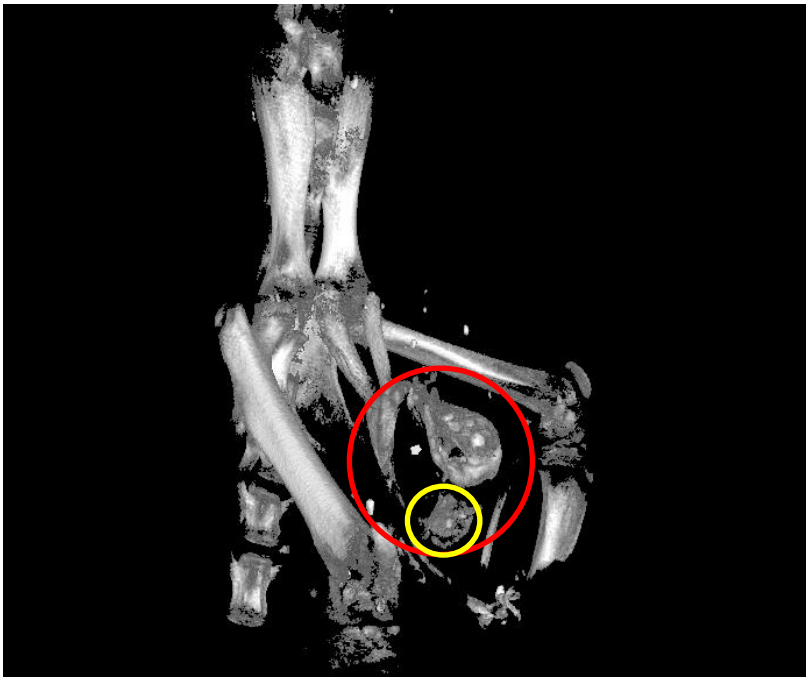

Figure 6.9 Day seven, CT image of the angled side view of the rat skeleton and silver nitrate in the bladder and urethra (circled in red). Possible leaked silver nitrate (circled in yellow).

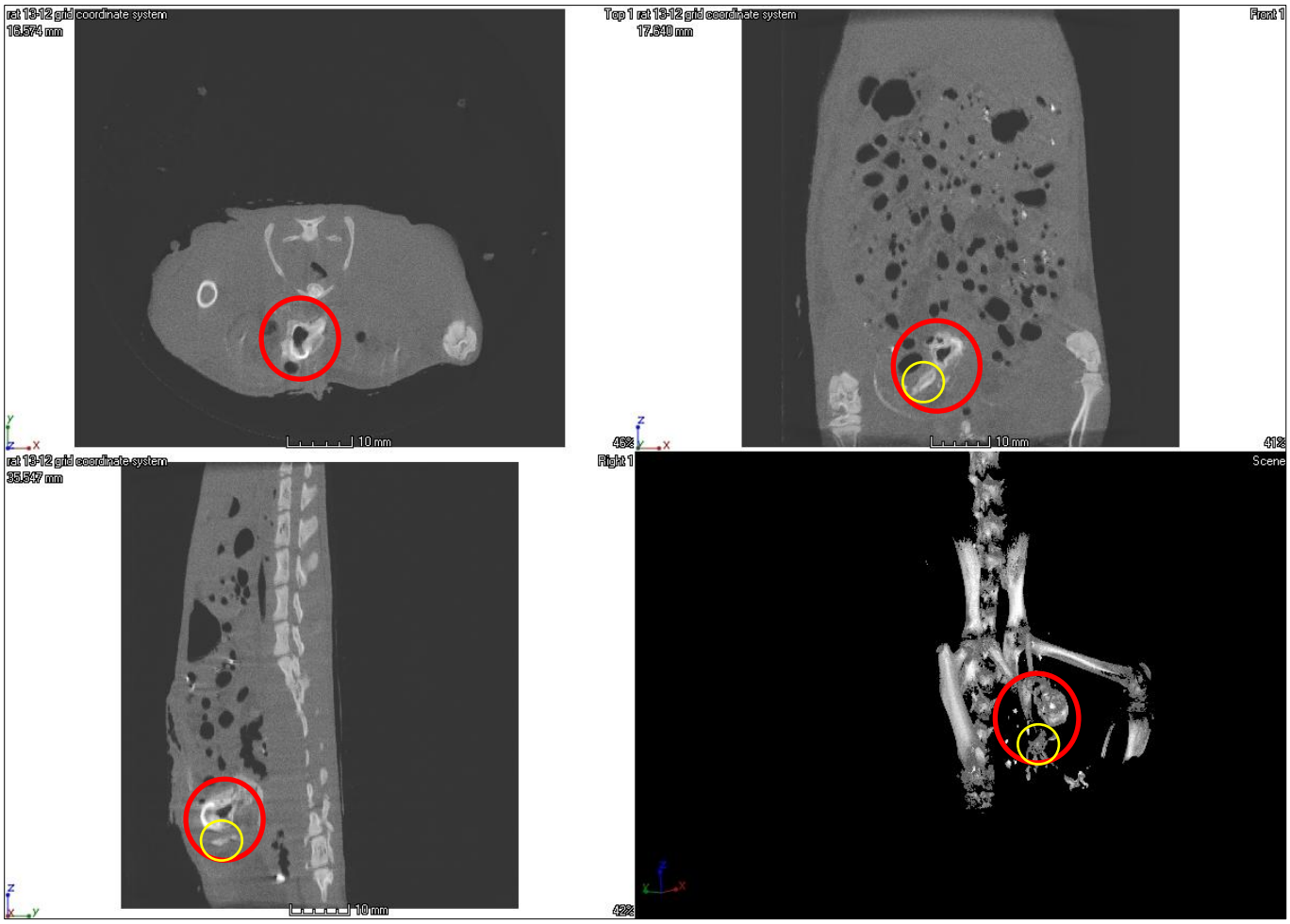

Figure 6.10 Day seven, top view (top left image), front view (top right image), left side view (bottom left image) of the abdomen of the whole rat including tissue, the scaled back image of the skeleton (bottom right image) and the silver nitrate within the bladder (circled in red). The possible leakage of silver nitrate, circled in yellow.

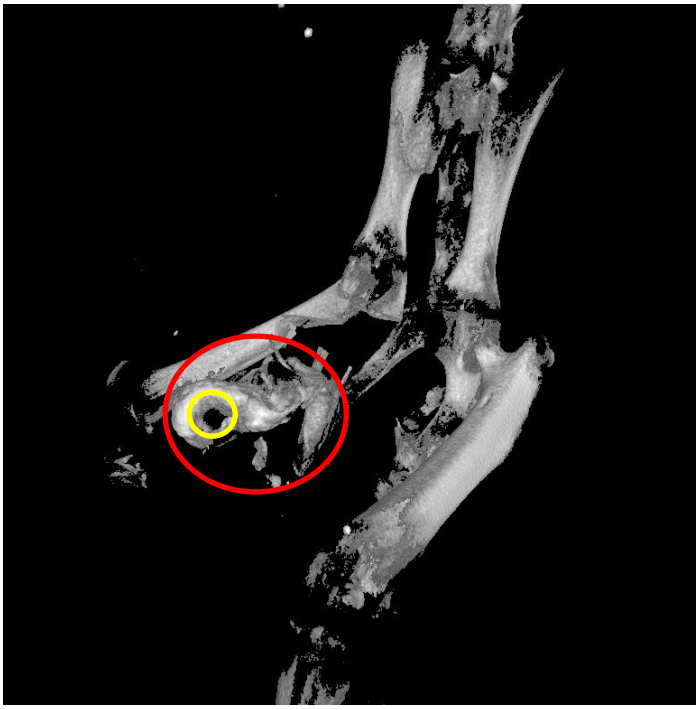

Figure 6.11 Day eight, CT image of the angled side view of the rat skeleton and silver nitrate in the bladder and urethra (circled in red). Air bubble (circled in yellow) within the bladder is visible.

Figure 6.12 Day eight, top view (top left image), front view (top right image), right side view (bottom left image) of the abdomen of the whole rat including tissue, the scaled back image of the skeleton (bottom right image) and the CT drug, silver nitrate, within the bladder (circled in red).

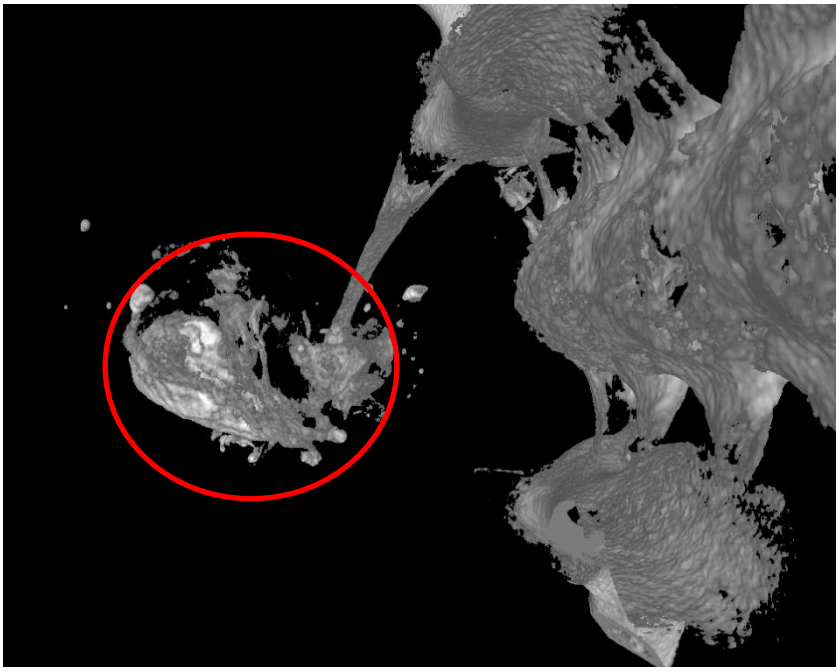

Figure 6.13 Day nine, CT image of the top view of the rat skeleton and silver nitrate in the bladder (circled in red)

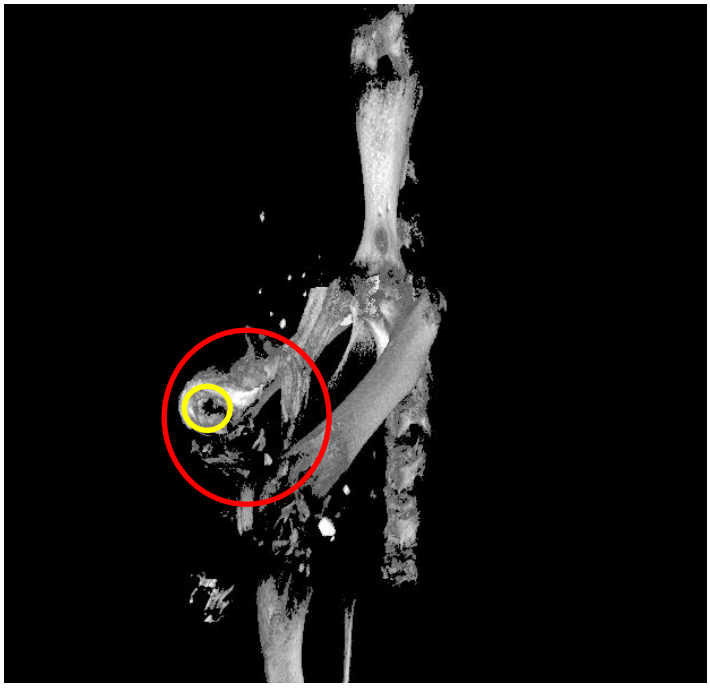

Figure 6.14 Day nine, CT image of the left side view of the rat skeleton and silver nitrate in the bladder and urethra (circled in red). Air bubble (circled in yellow) within the bladder is visible.

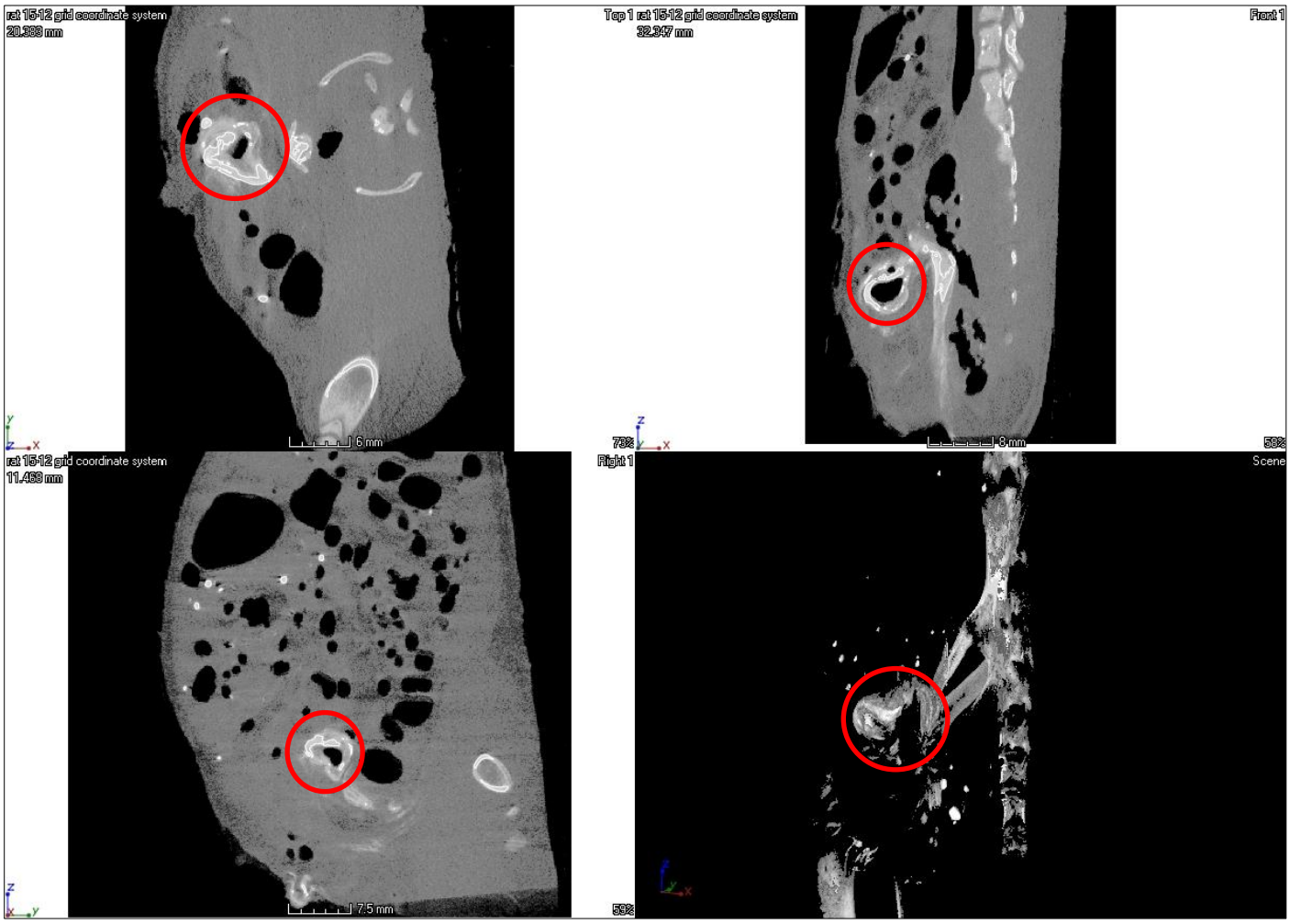

Figure 6.15 Day nine, top view (top left image), left side view (top right image), front view (bottom left image) of the abdomen of the whole rat including tissue, the scaled back image of the skeleton (bottom right image) and the CT drug, silver nitrate, within the bladder and urethra (circled in red).

6.4 Discussion

This section of the thesis focussed on the *in vivo* degradation of a rat bladder using CT imaging, which shows disruption occurred between day two and six including appearance of an air bubble in the bladder, possible silver nitrate leakage from the bladder and the shifting in bladder position further down the peritoneal cavity. The sixth day shows signs of leakage that became apparent from the right side of the bladder and there were larger air bubbles forming in the bladder (See Figure 6.7). The air bubbles show there is movement of the solution within the bladder, which could be due to absorption of the silver nitrate into the bladder tissue. The experiment was successful in relation to the CT drug used as this compound highlighted the bladder throughout the nine days (See Figure 6.13). The bladder shape shows to have changed, misshaping and moving lower into the peritoneal cavity, which could be due to the force applied to the bladder from the gases produced after death. In addition, the movement of the rat between the CT scanner and storage and the vertical position needed for scanning the rat could have participated in the bladder movement. The possible movement of the silver nitrate out and away from the bladder between day six and seven show that drug leakage and diffusion from the bladder is possible (See Figures 6.7 and 6.9). These results are consistent with the suggestions from the Japan case study (Moriya and Hashimoto, 2001) inferring higher drug concentrations in the femoral vein in comparison with cardiac blood could be due to drug diffusion from the bladder (Moriya and Hashimoto, 2001). This experiment was used as a model as the silver nitrate is structurally different from most drugs found within the bladder in post-mortem casework, therefore diffusion timelines will vary dependant on drug type and the physicochemical properties of the drug. However, the silver nitrate could not be seen on day eight, which could be due to dispersion and the concentration was too low to detect using the CT scanner. Therefore, it was not determined if the compound could reach the femoral vein within the nine day period, which could be rectified by increasing the concentration of silver nitrate inserted into the bladder.

This is an initial study into this area that has used a compound that has previously shown to be visible on the CT, which was the main priority for this experiment. However, these results would not be able to be used as a model for the antidepressant drugs due to different properties of silver nitrate. The molecular weight of silver nitrate is 169.87 g/mol, which is smaller than the average drug found in post-mortem cases and previous work has shown smaller compounds diffuse faster through the body (Zapata, Luna, 1989) meaning these results could be an accelerated version of drug movement over nine days. Ideally, a compound would be used that could be visible on the CT image and have similar chemical properties and therefore would behave in a similar way to the drugs that would usually be found within the bladder in post-mortem cases. In relation to using the rats as a model, these animals are much smaller than humans are and any diffusion observed would have to take into consideration any anatomical and size differences between humans and rats.

The basis of this experiment was to determine if there is bladder breakdown, if there was diffusion from the bladder and the distance the compound could diffuse within the nine days post-mortem. Specifically, would the compound reach the femoral vein in this period however, the femoral vein could not be highlighted as shown in section 3.3.5.4. In addition, there are no measurements to determine the distance the silver nitrate reached apart from a scale on the composition images. An improvement to this experiment would be to

identify the femoral vein and take specific measurements from different angles to determine the distance of the silver nitrate over time.

In comparison to the previous research carried out using post-mortem computed tomography (PMCT) this experiment has shown silver nitrate to be suitable for use to identify the bladder using Micro-CT over nine days post-mortem and also the possible movement of the compound out of the bladder could be due to leakage. However, this experiment is very limited with the use of one rat; repeats of this experiment under the same conditions would need to be carried out for reproducibility.

The silver nitrate (1M) could not be seen from day eight onwards, this could be due to the dilution of the compound once it is released from the bladder. The amount of fluid within the body is approximately 57-72% of the rats' body weight (Foy and Schnieden, 1960), which could be classed as a large volume for such a small amount of silver nitrate initially inserted into the bladder (0.25 ml). This range of values is the total body water, which can be calculated if the weight of the rat is known (192.81 g). The volume of fluid would be used to determine the dilution of the silver nitrate assuming all the solution leaked into the peritoneal cavity. However, a limitation with this model is the dilution would be overestimated as the total body water describes the compound diluting into the rat as a one-compartment model instead of where the compound could actually reach, the smaller volume around the bladder and lower peritoneal cavity. This model is based on the rat however; the calculations could be scaled up to include humans. The total body water is lower for humans with 60% for males and 50% for females (Levine, 2013) and the average weights are 83.6 kg (male) and 70.2 kg (female) (Office, 2010). In addition, the bladder volume of a human is much higher than a rat with differing bladder volumes dependant on gender, with bladder volumes of 351.3 ± 86.0 ml for men and 325.0 ± 76.7 ml for women (Kanyilmaz, Calis, Cinar, 2013).

In relation to the rat, the bladder contained 0.25 ml (experimental value) solution of 1M silver nitrate and would leak into between 110 ml or 139 ml of fluid in the peritoneal cavity. This leakage would cause the compound to be diluted and the resulting concentration could be calculated using $C_1V_1=C_2V_2$. There is a difference between the rat and human dilutions of silver nitrate with higher concentrations in the humans, which could be due to the small volume inserted into the rat, which was < 1 ml as the bladder membrane is not elastic after death reducing the amount of volume inserted into the bladder (See Table 6.1).

Table 6.1 The range of the total body water values from the range of percent of water found in rats and humans and silver nitrate dilution from these two values in the two species

	Rat	**Human**
Total Body Water (g)	109.81 (57%), 138.82 (72%)	50,160 (Male), 35,100 (Female)
Silver nitrate concentration (diluted) (M)	0.0023 (57%), 0.0018 (72%)	0.0070 (Male), 0.0093 (Female)

The results of this experiment show that after six days there is a possibility of leakage from the bladder. Therefore, if an individual is found within the six-day period there is a high possibility there is no release of drugs from the bladder based on this model. In addition, further work would need to be carried out using

higher concentrations of silver nitrate to determine if the silver nitrate can diffuse to the femoral vein and if this could be achieved within nine days. This is due to the disappearance of the compound from day eight due to the decrease in concentration of silver nitrate due to dispersion into the peritoneal cavity. Furthermore, alternative methods would need to be developed to highlight the femoral vein for measurements to be taken to determine how far the drug can diffuse over the nine days. This could then create a model showing if it is possible for drugs to diffuse to the femoral vein and alter drug concentrations to the point that could affect the outcome of cause of death. In addition, human models could be scanned using medical CT scanners subject to ethical approval.

Chapter 7 – Final Discussion and Future Work

7.1 Discussion

The drug concentration at the time of death can be used to determine whether the drug was in the therapeutic or toxic range, which would indicate if this drug could have been involved in the cause of death. However, the post-mortem samples taken at autopsy containing the drugs cannot be assumed to be the concentrations present at the time of death. Between death and the autopsy, the PMI, changes occur within the body that can alter the drug concentrations detected. PMR is one of these processes, which is the process that does not use energy, a passive process, involving the movement of drugs after death. PMR can lead to changes in drug concentrations at sampling sites after death. A number of organs including the lungs, liver, and heart are thought to be drug depots to "enable" PMR (Anderson and Jones, 1990; Prouty and Anderson, 1990). One possible depot for PMR is the bladder. To date there has been limited research on possible PMR from the bladder. Post-mortem redistribution is a wide area that affects numerous organs to differing extents. This phenomenon has been demonstrated in the heart, lungs, stomach, liver and even leg skeletal muscle (Pounder, 1997). However, limited research has been carried out in relation to PMR from the bladder. As a result, this research has focussed on this organ to fill the gap in toxicological research and also to develop models to study it further. The investigation focussed on the influence of bladder degradation, pH, urine volume, and temperature on diffusion from the bladder. *In vitro* diffusion through sections of the bladder and whole bladders, also *in vivo* diffusion from the bladder in rat models over 9 days.

The initial sets of experiments investigated the effect of a number of different parameters on the cumulative concentration of three different compounds (rhodamine B, amitriptyline and nortriptyline). The experiments used porcine bladder sections (sections from different bladders, inter-bladders and sections within the same bladder, intra-bladder) including temperature, pH, bladder tissue thickness (predicted to influence diffusion from the work by Fick) and bladder degradation. The main parameters that affected the cumulative concentration of the drugs was temperature however, this was limited to a significant difference between physiological temperature (37°C) and the two lower temperatures (20°C and 5°C). The post-mortem times where the significant differences occurred were at approximately 94 ± 0.1 hrs for pH 7.4 ($p < 0.001$) and 100 ± 0.06 hrs for pH 5 ($p > 0.0001$). The lower the temperature the less difference between the drug concentrations therefore, after the initial cooling stages of the body, or if the body was outside in cold temperatures, and after the body has been transferred to the mortuary the diffusion of drugs is reduced. This temperature related reduction in the diffusion has previously been found in the stomach (Yonemitsu, 1991; Smith, 1995). The pH had more of an effect on the two antidepressant drugs as there were results for all three temperatures at pH 7.4 and only one set of results at pH 5. This is possibly due to the higher percentage of ionisation of both the antidepressant drugs at the lower pH reducing the unionised concentration available to diffuse through the bladder tissue. The bladder degradation, which involved using fresh bladders for one set of experiments and thawing bladders that were frozen for one week prior to analysis, did not have an effect on the drug concentrations. The peak concentration for the fresh bladders at pH 7.4 and 37°C was 1.70 ± 1.0 mg/L and 2.63 ± 2.46 mg/L for degraded bladders. However, at pH 5 the higher concentration was reversed with the peak concentration of 2.29 ± 1.22 mg/L for the fresh bladders and 1.45 ± 1.26mg/L for the degraded bladders at 37°C. This shows that individuals found after a longer PMI, resulting in more degraded tissues, would not affect the amount of drug diffusing through the bladder tissue in cases where there is no damage to

the bladder producing openings. The peak concentrations of rhodamine B using fresh bladder at pH 7.4 sections were 1.70 ± 1.0 mg/L at 37°C, 0.67 ± 0.71 mg/L at 20°C and a concentration below the limit of detection of the method at 5°C. The fresh bladder sections at pH 5 and resulting rhodamine B concentrations of 2.29 ± 1.22 mg/L at 37°C, 0.85 ± 0.89 mg/L at 20°C and the amount of rhodamine B at 5°C was below the methods limit of detection. Similar results were found for the degraded tissues, further confirming tissue frozen and thawed, mimicking degraded tissue, may not affect drug concentrations diffusing from the bladder. However, the intra-bladder experiments had no clear trend with higher concentrations at lower temperatures (2.73 ± 2.31 mg/L at 20°C and 1.86 ± 1.75 mg/L and 37°C). There was an overall increase in rhodamine B over the course of the experiments however, there was also a large standard deviation reducing the difference between the lower temperatures. These results are to be expected as dealing with biological tissue, there will be variations due to the tissue itself resulting in outcomes that would not be exactly repeatable each time even when using the same conditions.

In relation to the drug ionisation, the pH showed more of an effect on the cumulative concentrations of the two antidepressant drugs (amitriptyline and nortriptyline) than the rhodamine B. The ionisation of rhodamine B at the two pH values was 99.9% in pH 7.4 and 86% in pH 5, which does show a difference however, this did not affect the amount of drug that diffused through the bladder tissue to any significant amount. Specifically for the antidepressant drugs, there were more results for the higher pH, which could be due to the change in ionisation state of the drugs. However, there was a smaller difference in ionisation for amitriptyline and nortriptyline with 99% and 99.5% at pH 7.4, 99.99%, and 99.99% in pH 5 respectively and a larger difference in results. This shows pH is more of a factor with the antidepressant drugs than the model compound, due to the small changes in the ionisation state of the antidepressant drugs. However, the results at pH 7.4 show approximately double the concentration of rhodamine B with peak cumulative concentrations of 6.69 mg/L and 6.69 ± 4.76 mg/L for amitriptyline and nortriptyline respectively. Lower concentrations at pH 5 were shown to be 1.96 mg/L and 2.8 ± 0.32 mg/L for amitriptyline and nortriptyline respectively therefore, the longer the PMI and the lower the pH in the bladder the fewer drugs would diffuse from the bladder. Most of the drugs found in post-mortem cases are weak bases and therefore may follow a similar trend as these results as they would possibly be mainly ionised in the urine after death and less likely to diffuse from the bladder unless there were holes in the bladder. However, even with the drugs being mostly ionised (86.0 % - 99.9 %) diffusion is still possible from the bladder over five days post-mortem. These results can be used as a model for other drugs as the Henderson-Hasselbalch equation could be used to determine the ionisation of the drug and use this as a guide to determine how much drug could possibly diffuse from the bladder. Other basic and acidic drugs may also have similar results, even with variable pKa values, as the acidic drugs with lower pKa values would be ionised at higher pH (pH 7.4 and 5) and the basic drugs with higher pKa values would also be ionised as the pH values for these experiments are generally lower. The difference in the concentration of these different drugs that would diffuse through the bladder tissue would depend on the other chemical and physical properties of the drugs. As stated previously, a number of parameters would need to be considered to make an accurate hypothesis of differing drugs and not determine from only one variable.

Tissue degradation would occur after death due to autolysis and putrefaction processes making up decomposition and the longer the PMI the more tissue degradation. This set of experiments investigated if there was a difference between fresh bladders mimicking close to time of death and frozen then thawed bladder tissue mimicking bladder tissue after a longer PMI of one week at cold temperatures. The tissue degradation was not a significant factor in the drug concentrations as there was no difference between the fresh and thawed bladder tissue for the two higher temperatures and both pH, there was differences between the lowest temperatures however, the concentrations were mostly below the limit of detection (0.16 mg/L).

The other factors that were investigated included the change in the donor chamber over the course of the experiment and if the decrease in the donor chamber was matched with the concentrations in the acceptor chamber. However, the results showed a large difference between the initial donor chamber concentration and the combined final concentration of the donor and acceptor chamber, which shows that the drugs have a high affinity for the bladder tissue. The largest difference was 89.62 mg/L (fresh bladders, 37°C, pH 7.4) and the smallest difference was 11.47 mg/L (intra-bladders, 20°C, pH 7.4). This is possibly due to the lipophilicity of the drugs all with log P values of 4.92 (amitriptyline) (Hansch, Leo, 1995), 4.51 (nortriptyline) (Brodin, 1974) and 2.43 (rhodamine B) (Mah *et al.*, 2013). Another parameter was the bladder thickness, this was measured for all the *in vitro* experiments and the statistical results showed there was a small spread of values between 4.48 – 5.0 mm with no significant differences between the fresh and degraded bladders (p 0.42) and within the same bladder (p 0.23). Therefore, for this set of experiments due to the similarity of the bladder thickness this was discounted as a possible factor that could affect drug diffusion through bladder tissue. Finally, the acceptor chamber pH was recorded at the start and end of each experiment and the pH 7.4 experiments decreased (Fresh inter-bladder, 37°C, pH 7.4, final pH 6.87 ± 0.24) during the experiment. The pH 5 resulted in an increased pH (fresh inter-bladder, 37°C, pH 5, final pH 6.95 ± 0.16), which is consistent with the start of autolysis and the start of putrefaction.

The permeability values of rhodamine B that were calculated showed the higher temperature had ten times the magnitude of permeability, 1.8×10^{-4} cm/s at 37°C, in comparison with the two lower temperatures, which had similar permeability values, 6.8×10^{-5} cm/s for 20°C and 1.4×10^{-5} cm/s for 5°C. This shows as the temperature increases the permeability increases and a higher concentration of the drug can diffuse through the bladder tissue as expected by the work of Fick (Fick, 1995). The permeability of rhodamine B was also not affected by the degraded tissue resulting in similar permeability values at 37°C. Fresh tissue values were 1.8×10^{-4} cm/s (pH 7.4) and 1.8×10^{-4} cm/s (pH 5) and for degraded tissue at 3.6×10^{-4} cm/s (pH 7.4) and 1.4×10^{-4} cm/s pH 5 at 37°C. This was used as an example however; the higher temperature with degraded tissue may not be possible, as the body would have cooled before significant tissue degradation would have begun. The intra-bladder results showed that if physiological pH were maintained the permeability would not be affected until the body cooled to below room temperature, 3.0×10^{-4} cm/s at 37°C and 3.5×10^{-4} cm/s at 20°C. However, the permeability would most likely reduce as the pH reduces after death, which results in less drugs diffusing from the bladder after the body temperature reduced from 37°C. This is consistent with the results showing the lower pH produces lower permeability values and therefore, the longer the PMI the less drugs are able to permeate though the bladder tissue, which is possibly due to the ionisation of the drugs. The

antidepressant drugs had the same magnitude for all the results including pH 7.4 (37°C, 20°C and 5°C) and pH 5 (37°C). A previous study investigating amitriptyline diffusing through Caco-2-cells in pH 7.4 at 37°C resulted in a permeability of 2.1 x 10^{-5} cm/s (Faassen *et al.*, 2003). This research produced a value of 1.9 x 10^{-5} cm/s under the same temperature and pH conditions, these results show amitriptyline being a highly permeable compound. The more polar metabolite, nortriptyline, had permeability values that were slightly lower than amitriptyline with 3.0 x 10^{-5} cm/s (pH 7.4) and 4.6 x 10^{-5} cm/s in pH 5. This could be due to the polar nature and the 99.9% ionised form of the drug. All three drugs had higher permeability at physiological temperature therefore, closer to the time of death there would be highest amount of drug diffusion, which would theoretically decrease as the body temperature decreased.

The results show that there is diffusion from the bladder sections at the higher temperature and pH; however, this was not mimicking the human element of this research, as the bladder would be intact. Therefore, this was the reason of using the intact bladder to determine if the results from the bladder sections were consistent with the intact bladder. In addition, the extension to the research was used to determine the better "model" for further study.

The *in vitro* experiments involving the intact bladders resulted in the volume being more of a factor than concentration that would affect the amount of rhodamine B diffusing through the bladder tissue within the first 100 hours post-mortem. The lower initial concentration of rhodamine B resulted in higher acceptor chamber cumulative concentration showing concentration is not a factor in diffusion. The peak concentration of rhodamine B was 3.5 ± 1.02 mg/L at 100 mg/L using pH 7.4 at 20°C. The volume did have differing results showing full bladder had increased diffusion over half-filled bladders with cumulative concentrations of 3.5 ± 1.02 mg/L and 0.95 ± 0.73 mg/L respectively, which shows that if the bladder is not voided after death there could be more drug diffusion. However, this is not always the case and therefore this would reduce the amount of drugs diffusing from the bladder. It has been stated that in approximately 50% of cases the bladder is voided at time of death (Negrusz, 2013) and therefore urine is not always available. From the results of this research, the reduction of diffusion from the bladder is reduced with lower temperatures, which would occur when the body is stored in the mortuary fridge. However, due to the variable times of PMI before the individuals arrive at the mortuary, and to prevent any further diffusion, the bladder should be emptied upon arrival at the mortuary. The pH shows to have an effect on the cumulative concentration of rhodamine B with significant differences ($p < 0.0001$) between pH 7.4 and 5. The consistently higher cumulative concentrations of rhodamine B at pH 7.4, with the pKa of 4.2, result in a 99.9% ionised drug that is consistent with results from a previous study describing due to the hydrophilic layer on the lumen of the bladder ionised drug preferentially diffuse through the bladder (Moch, Salmon and Armesto, 2014). However, this is only evident at the later stages of the experiments, approximately 97 hours, and therefore this could more likely be due to bladder degradation. The tissue could have degraded and caused openings in the tissue, which could have occurred at a quicker rate due to the increased pH. The tissue was not specifically checked for openings at the end of the experiment and so could not narrow down this line of investigation any further at this time. Furthermore, this pH would not be evident at the later stages of decomposition as the pH decreases and would be showing similar amounts of drug concentrations found at pH 5.

A limitation of this experiment was the inability to measure the bladder thickness, even though from the previous chapter this was shown due to the similarity of the bladder thickness it was not a factor in those specific experiments. The bladders used were in different states including empty and contracted bladders and bladder filled with urine and therefore there was a wide difference in bladder size and as a result bladder membrane thickness. On the other hand, even without the measurements using a number of bladders with differing volumes would produce a robust model that would mimic the conditions for deceased individuals.

The time scale of the work by (Moriya and Hashimoto, 2001) was nine days, this gives a reference point for this research using a real work example where post-mortem diffusion from the bladder was suspected. The initial point was it took approximately 97 hrs for rhodamine B to diffuse from the bladder to the cumulative concentration of 3.5 ± 1.02 mg/L. This concentration would be increased if this concentration was the initial concentration inside the bladder in 5 ml of solution to 6.72 ± 36.72 mg/L (average of male and female peritoneal fluid rhodamine B concentrations), which was determined from the volumes of peritoneal fluid, differing for males and females, outside the bladder. An unknown section to this research would be the dilution factor and the time taken for the drug to diffuse from the bladder to the femoral vein. There is a range of times for the drug to diffuse through the femoral vein and then increase the drug concentration within the vein. Overall, the length of time from death to the drug potentially increasing the drug concentration in the femoral vein would be longer than 179 – 197 hrs post-mortem. An extension to this section of the research was to move onto *in vivo* work which would give a visual representation of the actions of the drug within the bladder and the possibility of the drug diffusing to the femoral vein within the suggested nine days post-mortem (Moriya and Hashimoto, 2001).

In order to try to visualise the movement of any drugs an *in vivo* based experiment, investigated diffusion from the bladder using μx-ray CT, with sliver nitrate as the contrast reagent within a rat. The results showed movement of the bladder location to further down into the peritoneal cavity and also the degradation of the bladder to a point where there was leakage of the silver nitrate from the bladder between day two and six. The movement of the bladder could be due to gravity as the rat was vertical for scanning and transported between storage and the CT scanner. There was displacement of the silver nitrate solution as air bubbles were formed in the bladder, which could be due to the movement of the bladder, diffusion of the solution into the bladder membrane and the eventual leakage of some of the silver nitrate solution from the bladder. This bladder breakdown between day two and six shows there is a possibility for there to be drug solution leakage from the bladder and this would then be in the peritoneal cavity and able to diffuse freely through the tissues and possibility contaminating sampling sites. The Japanese study had consistent results with this research with the suggestion of possible drug movement from the bladder (Moriya and Hashimoto, 2001).

However, silver nitrate is smaller and structurally different from the drugs usually found in post-mortem casework and therefore the diffusion of this drug may not mimic these drugs, this was used in a similar way to rhodamine B in the *in vitro* studies as a model compound. This drug was successful in relation to the visibility on the CT until day eight where it disappeared, which was possibly due to the concentration of silver nitrate

decreasing below the LOD of the CT upon release from the bladder. As a result, the ultimate aim of determining if the silver nitrate could reach the femoral vein was not executed. In addition, this could not be completed, as the femoral vein could not be successfully identified on the CT without reducing the resolution for the bone and silver nitrate, as these were indistinguishable with the introduction of the alloy fuse wire. This section of the research would need more investigation to complete the aim of the in-vivo research as the drug was looked at in relation to diffusion from the bladder, it also needs to be determined if the drug can reach the femoral vein. The dilution of the silver nitrate was determined for both the rat and scaled up for humans using the total body water to determine the concentration that could not have been seen on the CT scanner. The reduction in concentration were overestimated as this total body water is not limited to the peritoneal cavity where the silver nitrate would most likely have been able to reach, it includes the total amount of water in the body. The range of concentrations was 0.0018 – 0.0023 M for the rat and scaled up to humans with a dilution to 0.0070 M for males and 0.0093 M for females. Therefore, if an individual were found within six days after death there would not be a concern with a large amount of drug leakage from the bladder and therefore limited chance of the drug diffusing into the femoral vein and altering the drug concentration within that specific sampling site.

Overall, the bladder was intact for two days after death; further studies would need to be carried out to determine if this is repeatable. It is interesting to note that in all the *in vitro* studies the bladders remained intact for the duration of the experiments. There is a small amount of diffusion of drugs through the bladder tissue within the first five-days and leakage from the bladder on the sixth day. As a result, based on the results of this study the bladder is not likely to be a significant drug depot for post-mortem redistribution for amitriptyline and nortriptyline for the first five days after death. The concentrations resulted from this research would not be able to alter the drug concentrations within the femoral vein to a significant enough degree that could alter the toxicological interpretation of a case.

7.2 Future Work

This research was based around the investigation of the most appropriate method for determining the amount of drug diffusing through bladder tissue after death over a 9-day period. The initial experiments have produced a robust method however; there can be improvements to build on the current method both in relation to the experimental method and the types of sample solutions and drugs used in the experiments. This line of thinking can also be applied to the *in vivo* experiment, where there can be a number of improvements in the experimental method and alterations to the conditions the rat could be stored under during the experiment.

The first study involved the Franz cells and this method was significantly developed and a robust method was produced, which allowed for the sealing of bladder tissue onto the Franz cells and the ability to run the experiment for 100 hours. However, the solutions used to dissolve the drugs were buffer, which is useful for stabilising the pH for the initial experiments. To further this aspect of the research the solutions could be changed to synthetic urine, which would mimic the conditions found after death in deceased persons, as urine would be present in the bladder and this would allow for more realistic conditions and pH values. A known concentration of the drug would be dissolved in the synthetic urine and these solutions would be placed into the donor chamber of the Franz cells. The synthetic urine would be checked for pH values before and after the experiment to determine any changes and this could then be used to determine how this affects the ionisation state of the drug and link this to the amount of drug diffused through the tissue during the experiment. Furthermore, the drug concentrations themselves could be taken further by using both expected therapeutic concentrations found in the urine and toxic concentrations, which would mimic both adherence to taking prescribed medication and instances of overdose. The Franz cell experiments were conducted at set temperatures, physiological (37°C), room temperature (20°C) and mortuary fridge temperature (5°C). However, after death the body does not stay at a fixed temperature the body cools down and therefore to advance the experiments a water bath with a gradient temperature system would be used. The water bath would be set to the cooling rate of the body to determine how this would affect the amount of drug diffusing through the bladder tissue over the post-mortem interval of 100 hours. In addition, the permeability coefficients of each drug could be used in further work involving calculating the diffusion coefficient, which could then be used to determine the time taken for each drug to diffuse to set distances (Clark, Edeson and Ryall, 1983). This would be useful in relation to calculating the time taken for a drug to reach the femoral vein from the bladder, measurements between the bladder and the femoral vein would be needed for these equations.

Similar changes would be introduced for the next set of experiments using the intact bladder where synthetic urine would be used for the drug solution. The temperature would be closely monitored to mimic the cooling of the body the intact bladders could be used to test the antidepressant drugs in a similar manner to the bladder section experiments. Another aspect of both these experiments that could be expanded would be the types of drugs that were being tested. The two antidepressant drugs used for the first set of experiments were chosen, as they were found in numerous post-mortem cases and so relevant for the toxicology community to determine how these drugs would act after death in relation to the diffusion through bladder tissue. However,

there are a number of different types of drugs that are found in post-mortem cases and have differing physical and chemical properties and as discussed previously drug properties are a factor in the effect on the ionisation, which in turn affects the amount of drug available to diffuse through biological tissue. Therefore, expanding the number of drugs usually found in urine in post-mortem cases in the diffusion experiments would give a better understanding of how these drugs would act after death if present in the bladder. Furthermore, other possible experiments include using multiple drugs that would be found in conjunction with each other in the bladder would be added to the diffusion chamber to see if the presence of multiple drugs could affect the diffusion of each drug individually.

A different aspect of the *in vitro* based research could be investigated based on the tissue binding of the drugs to different organs and vessels including the bladder, intestines and the femoral vein. This would allow determining if certain drugs have a higher affinity for the tissue and if this would significantly affect the results produced from the blood samples used to determine drug concentration for toxicological interpretation.

The *in vivo* study was the most recent addition to the research and therefore less work was carried out, a number of aspects of the method were developed including the catheterisation of the rat with a drug able to be visible on the CT and the rat was secured for scanning and stored during the experiment. However, only one rat was analysed using this method for the full duration of nine days, this experiment would need to be repeated to determine if there is any variation between at least three repeats. As each rat would be slightly different and this could affect the diffusion of the drug, as it was found after death the bladder loses elasticity. After the variable volume of urine was removed, the bladder does not reduce in size and as a result, the bladder can hold differing volume of solution that is replaced in the bladder for the experiments. The rat was not scanned each day which left a gap in the results, ideally the rat would be scanned each day to get a full picture of the bladder changes over the nine day period. Furthermore, this variation in size also affect the bladder thickness, the more urine in the bladder after death the thinner the bladder walls, which was a factor that was looked into as a possible factor that could affect the amount of drug diffusion through the bladder tissue.

Another issue was with the drug itself, even though the drug (silver nitrate) was visible for most of the experiment as it began to diffuse from the rat bladder it disappeared after day seven of the experiment, which could be due to the drug concentration. Therefore, a repeat of the experiment with an increased concentration of the drug could improve the visibility of the drug outside of the bladder enabling to determine where the drug diffuses in the peritoneal cavity. This would be useful to determine if the drug could diffuse to the femoral vein, which was the ultimate aim of the experiment. A further set of experiments would be to change the drugs to those found in casework and radiolabel these drugs to be able to be visible on the CT as these are the drugs that are relevant to the toxicological community.

Future work would include repeating this experiment at a temperature that would mimic the case report from 2001 (Moriya and Hashimoto, 2001) as the individual was found outside in winter, these conditions would result in lower temperatures than 20°C. Therefore, the lower temperature of 5°C would be used to compare to

room temperature to determine if the lower temperature would slow down bladder break down *in vivo*. Furthermore, methods for measuring the distance of the drug diffusion could be incorporated to allow for rate calculations, which could then be used as a model for comparison with other types of drugs to determine the possibility of the drug diffusing to the femoral vein within nine days after death. This would be useful for individuals found within this timeframe. Another type of *in vivo* experiment could be using larger animals, specifically pigs, due to their similarity with humans. A known amount of drug could be administered and after death the femoral vein could be sampled at set times over a period. In addition, the bladder could be catheterised using a known concentration of drug and the femoral vein would be sampled at set times over the nine day period, suggested by the Japanese paper (Moriya and Hashimoto, 2001), to determine if the drug could be detected during this period. The pigs would be a closer mimic to humans due to ethical restrictions however, it would be ideal to use human cadavers to mimic the drug movement after death.

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