

The influence of plasma proteins biology essay

[Science](#), [Biology](#)



Introduction: With the pharmaceutical industry desperately looking to cut drug development costs, accurate and effective in vitro techniques used for pharmacokinetic profiling are of utmost importance. This project uses the local anaesthetic bupivacaine as a test compound to investigate how the binding of plasma proteins to drugs influences the availability of drugs to cross biological membranes. In addition, gaining insight to the molecular forces that govern the binding will also be investigated. Aims and Objectives: To confirm the binding between bupivacaine and the test proteins and to determine the specific molecular interactions responsible for these interactions. Another main aim is to investigate the influence of proteins on bupivacaine's ability and availability to diffuse across a semi-permeable membrane. This was achieved by determining binding parameters such as dissociation constants and percent binding. Methods: Fluorescence spectroscopy was used to confirm the binding between bupivacaine and the test proteins (BSA and AAG). This method was also employed to determine the molecular forces that govern this binding using sodium chloride in universal buffer. Equilibrium dialysis was the method used to separate free and bound bupivacaine while UV-spectroscopy was used to quantify the unknown bupivacaine concentrations. Results: Fluorescence spectroscopy confirmed weak binding between BSA and bupivacaine and even weaker binding between AAG and bupivacaine. The addition of sodium chloride in the fluorescence experiments determined that the binding observed is mainly mediated by electrostatic forces with both proteins. Equilibrium dialysis experiments revealed much stronger binding between BSA and bupivacaine and the use of UV-spectroscopy to quantify unknown

bupivacaine concentrations yielded results very similar to that in the literature. Conclusion: Binding does occur between bupivacaine and both BSA and AAG. Generally, protein binding does influence the transport of bupivacaine across a semi-permeable membrane by reducing the amount that is available to diffuse. Lastly, using UV-spectroscopy to quantify the results of equilibrium dialysis may not be an effective and comparable method to other quantifying techniques. Significance: The pharmaceutical industry may look to integrate the binding techniques used in this research to better understand how drugs bind to in vivo plasma proteins. This will aid in building more accurate pharmacokinetic profiles for drugs and will eliminate compounds with poor pharmacokinetic profiles that are likely to fail in later developmental stages, thus reducing costs.

Introduction

Novel Drug Development

Novel drug compounds must go through a rigorous process before reaching consumer shelves. The process from lead compound discovery to drug market approval is a complex, expensive and time-consuming one. The average time taken for the development of a novel compound is approximately 10 to 15 years [1]. The complexity of this process becomes evident when attempting to develop a compound that exhibits both desirable pharmacokinetic and pharmacological properties. For this reason, the pharmaceutical industry spends approximately 800 million to 1 billion dollars for the successful development of a market approved drug [1]. This enormous cost includes thousands of failed lead compounds that do not make it to later developmental stages [1]. This high attrition rate is a major

problem facing the pharmaceutical industry today and is demonstrated with an 11% success rate of marketed drugs [2]. According to Avdeef, A, 30% of lead compound attrition is due to pharmacokinetic failures [3]. In order for these statistics to change, pharmaceutical companies must focus their time and resources into developing pharmacokinetic techniques that will effectively screen lead compounds and eliminate others that are likely to fail in later - more expensive - developmental stages, such as clinical trials. Methods such as combinatorial chemistry and high throughput screening (HTS) have been at the forefront of novel drug discovery in recent years [4]. HTS allows for many lead compounds to be screened against potential targets in a simple, cost effective and automated manner, while combinatorial chemistry generates a large number of structurally diverse compounds [5]. With pharmacokinetic profiling being as important as it is in reducing attrition rates, this project will focus on the binding of bupivacaine using in vitro protein binding techniques.

Bupivacaine Hydrochloride

Structure and Chemistry

Local anaesthetics typically fall into two categories; the aminoamides or the aminoesters. Bupivacaine which is categorized as an aminoamide contains a lipophilic aromatic ring and a hydrophilic tertiary amine which is connected by an amide linkage as shown below in figure 1 [6]. Figure : Structure of bupivacaine containing an aromatic ring, a hydrophilic tertiary amine connected by an amide linkage [7] Bupivacaine hydrochloride comes as a white crystalline powder with a molecular weight of 342. 9 [7]. In terms of solubility, according to Clarke's Analysis of Drugs and Poisons, bupivacaine is

soluble in water (1 in 25) and ethanol (1 in 8). It is also known to be slightly soluble in chloroform, acetone and ether [7]. The dissociation constant (pKa) of this drug is 8.1, indicating it is a strong base [7]. Lastly, the partition coefficient (Log P) of this drug is 3.4 [7].

Pharmacokinetics

The pharmacokinetics of drugs refers to how the body handles the specific drug. To obtain a pharmacokinetic profile, the absorption, distribution, metabolism, and elimination are studied. The pharmacokinetic profile of bupivacaine is discussed below. Absorption: The absorption of bupivacaine is dependent on a few factors such as concentration, dosage of drug given, administration site and capillary blood flow to the administration site [8]. The peak plasma concentrations in caudal, epidural, and peripheral nerve block injections were seen at around 30-45 minutes [9]. Distribution: Bupivacaine, like most local anaesthetics is widely distributed throughout the body. The volume of distribution of this drug is approximately 1L/kg [7]. Bupivacaine is about 90% bound to plasma proteins. Therefore, the remaining proportion of unbound drug (10%) is able to be distributed freely and exert its pharmacological effect throughout the body [7]. Metabolism: According to Clarke's Analysis of Drugs and Poisons, bupivacaine is metabolised in the liver by oxidative dealkylation and hydroxylation [7]. The oxidative dealkylation reaction converts bupivacaine its major metabolite 2', 6' - pipercoloxylidide while the hydroxylation reaction occurs on the 4' position [7]. This metabolite is then catalysed by the cytochrome P450 3A4 enzyme [10]. Elimination: The main route of elimination for Bupivacaine's inactive metabolites is through the urine [7]. Approximately less than 10% of a given

dose is excreted as unchanged drug in the urine [7]. The plasma clearance of bupivacaine is around 8 mL/min/kg [7].

Pharmacology

The mode of action of bupivacaine is well known due to the many years of studies on local anaesthetics. Bupivacaine, like other amide anaesthetics exerts its anaesthetic activity by blocking the generation and conduction of nerve impulses [11]. It blocks the conduction of nerve impulses by binding to active sodium channels on the nerve cell. This in turn reduces the permeability of the nerve cell membrane to incoming sodium ions which are needed for depolarization [10]. By reducing the rate of rise of action potentials, autonomic activity is affected first, followed by pain loss and other sensory functions, and lastly, loss of motor activity [10]. Figure 2 below shows that the uncharged local anaesthetic diffuses across the neuronal membrane and ends up in the cytoplasm. Once in the cytoplasm, the anaesthetic becomes protonated and is now able to bind intracellularly and block the voltage gated sodium channel [10]. Figure : Pathway of a local anaesthetic (B) like bupivacaine as the unionized form travels from outside of the cell into the cytoplasm where it becomes protonated (BH⁺) and blocks a sodium channel [10].

Indications and Administration

Bupivacaine is a local anaesthetic that is classified in the amide group of anaesthetics. It is indicated for use in local infiltration, peripheral nerve block, epidural block (lumbar surgery, caudal surgery, labour), sympathetic block, intrathecal anaesthesia, and dental anaesthesia [12]. Bupivacaine can

only be administered by injection or infusion. In the UK, it is only licensed in injection at the concentrations of 0.25% and 0.5% and in infusion at 0.1% and 0.125% [12]. With many local anaesthetics, the co-administration of adrenaline is commonly used in practise and bupivacaine is not an exception. This is simply due to the vasoconstrictor property of adrenaline that slows the systemic absorption of bupivacaine and hence prolongs the action of the anaesthetic in the local desired site [12].

Passive Diffusion

Concept of Passive Diffusion

The movement of drugs across biological membranes is highly regulated by passive diffusion. In many drug absorption and distribution studies conducted, the dominating process of drug transport seems to be diffusion [13]. This concept pertains to the movement of a drug from an area of high concentration to an area of low concentration, also termed the concentration gradient [14]. Passive diffusion is differentiated from other types of transport mechanisms by the fact that it depends only on this concentration gradient and does not require any energy expenditure [14]. This net movement continues until equilibrium is reached. At equilibrium, the concentration of free unbound drug is the same on both sides of the barrier membrane. This transport method is taken into account because the movement of bupivacaine across the membrane in the equilibrium dialysis experiments of this project is solely dependent on passive diffusion. It is important to note that the rate at which drugs diffuse across membranes is not only dependent on the concentration gradient, but the membrane itself [13]. The semi-permeable membrane will be discussed next.

Movement through Barrier Membrane

In order for a drug to reach its target and exert its desired pharmacological effect, it must first pass through barrier membranes. These membranes are strategically set up throughout the body and include the gastrointestinal tract as well as the blood-brain-barrier. They play a pivotal role in cellular function by separating the intracellular and extracellular environments [15].

Figure : Structure of a biological cell membrane [15] Cellular membranes are hydrophobic in nature due to the amphipathic phospholipids that make up their structure. A phospholipid is composed of a hydrophilic phosphate group at one end and two long fatty acid chains which are hydrophobic [15]. Figure 3 illustrates that the hydrophobic chains are orientated inwards while the hydrophilic 'heads' are facing the outer aqueous environment. In the equilibrium dialysis experiments, a methyl cellulose membrane will be used to represent in vivo membranes such as biological tissues and cellular membranes. It is important to note that only unbound and unionized drug is capable of diffusing across a semi-permeable membrane.

Ionization of Bupivacaine

Although the ionization of bupivacaine will not be studied in this project, it is still important to determine its ionization in the constant pH that will be used throughout the duration of this project. Since bupivacaine is basic, the following Henderson- Hasselbalch equation is applied. Equation : Percent protonation of a basic drug using the Henderson-Hasselblach equation [16] The pH of the universal buffer used throughout this project is 7. 4 and the pKa of bupivacaine as previously discussed is 8. 1. When the above equation is applied, bupivacaine is approximately 83. 4% protonated

indicating that approximately 16.6% of bupivacaine is unionized and able to diffuse across the semi-permeable membrane.

Protein Binding

Concept of Protein Binding

The concept of plasma protein binding is critical when analysing the properties of a drug. The binding of drugs to plasma proteins is responsible for many factors of a drug's pharmacokinetic and pharmacodynamic properties such as drug distribution, duration of action and efficacy [17]. Protein binding occurs when a drug is exposed to plasma proteins. This exposure can occur in two ways; directly into the blood plasma via injection or via absorption from the gut. Depending on the drug's affinity for these plasma proteins, some of the drug will bind to the plasma proteins setting up drug-protein complexes and some will remain unbound. Only the unbound fraction of the drug is able to diffuse from the blood and be distributed to other compartments of the body and exert its pharmacological effect [17]. It is important to note that most drugs bind reversibly to plasma proteins, so the drug-protein complexes [DP] can associate (k_1) and dissociate (k_{-1}) quite rapidly [17]. This process is illustrated below using the law of mass action [17]. Figure : the association and dissociation of a drug-protein complex [17] This reversible binding indicates that equilibrium between drug and protein and the drug-protein complex will eventually be achieved. More specifically, equilibrium is attained when the rate of new drug-protein complexes being formed equals the rate at which the drug-protein complexes dissociate. The association constant (k_a) represents the drug's affinity for the protein and can be calculated using equation 2. This equation

states that the association constant is equal to the inverse of the dissociation constant (K_d). The dissociation constant will be evaluated in this project using two experimental methods and will be compared. Equation : K_a is equal to the inverse of K_d [17] Lastly, the degree of protein binding can be calculated as a percentage using the free (C_{free}) or unbound drug concentration and the total drug concentration (C_{total}) [17]. Equation 3 represents this degree of binding. Equation : The percentage of plasma protein binding is equal to the concentration bound over the total concentration [17] This degree of binding can also be expressed as the fraction unbound in plasma using equation 4. Equation : Fraction of unbound drug in the plasma [17] Although very useful, the law of mass action must make a few assumptions in order to be used [17]. These assumptions include that all of the proteins are equally available to the drug, the binding does not alter or modify the drug or protein in any way, the binding is reversible, and lastly the protein is either free or bound to the drug [17].

Protein Binding of Bupivacaine

As previously stated, bupivacaine is 90% bound to plasma proteins [7]. In terms of the proteins involved in bupivacaine binding, human serum albumin (HSA) and alpha 1 acid -glycoprotein (AAG) are thought to be two of the most important human plasma proteins [18]. AAG is expressed in the plasma at a much lower concentration (0.04-0.1 g/dL) than albumin (3.5-5 g/dL) and is involved in normal physiological processes such as coagulation and tissue repair [12]. AAG has a molecular weight of 40,000 and is an acidic protein due to the high content of sialic acid [19]. Denson et al. conducted an experiment to further enhance the understanding of bupivacaine's

binding, specifically to albumin and AAG. The results of this study showed the binding of bupivacaine to AAG as high affinity binding, while the binding to albumin indicated low affinity binding [18]. However, many other proteins expressed in the plasma are capable of binding bupivacaine such as; α 1-lipoprotein, α 2-mucoprotein, and γ -globulin [20]. The highly lipophilic nature of bupivacaine as seen with a Log P of 3.4, indicates that it would favour binding to a lipophilic protein like α 1-lipoprotein [20]. Bovine Serum albumin (BSA) is a protein that has been studied and used widely in drug research for a number of years. Although BSA is easily accessible and inexpensive, the main reason for its use is due to the similar homology between BSA and HSA [21]. In terms of the composition of BSA, the critical characteristic that makes this protein useful is the two tryptophan residues that are capable of fluorescing [21]. This has proven extremely useful in drug-protein binding studies. The diagram below highlights these two residues. Full-size image (44 K)Figure 4: Bovine Serum Albumin with tryptophan residues highlighted [21]

The distribution of drugs in vivo is highly dependent on protein binding. Since one of bupivacaine's main clinical indications is epidural block during labour, Mather et al. conducted a study using different human plasma proteins in both maternal and foetal plasma. Generally, as the concentration of bupivacaine was increased, the amount of drug bound to albumin, α 1-lipoprotein, α 2-mucoprotein, and γ -globulin decreased in both foetal and maternal plasma [20]. The difference in plasma concentration of bupivacaine between maternal and foetal plasma was shown to be due to greater protein binding of the drug to the maternal plasma proteins [22]. This was confirmed in a study that showed that at a concentration of 1 mcg/mL of bupivacaine,

95% was bound to maternal plasma as opposed to 66% which was bound to foetal plasma [23]. Although varying the concentration of drug provides clear correlation between drug and protein binding in the literature, it is not the only effective method. Altering the protein concentration can provide useful insight as many diseases and drug variability between patients is due to either reduced proteins or overexpressed proteins in the plasma [18]. For example, the elderly are known to have higher than normal concentrations of AAG which if combined with other diseases can affect the binding of basic drugs such as bupivacaine [18]. Altering protein concentration as well as drug concentration will be investigated in this research to determine how the protein binding is influenced.

Determination of Bupivacaine's binding

Equilibrium Dialysis

Many techniques to determine protein binding have been adopted over the years such as equilibrium dialysis (ED), ultrafiltration, ultracentrifugation, gel filtration and fluorescence spectroscopy [24]. A study of the first four methods described above was conducted to test reliability by Kurz H et al. and concluded that equilibrium dialysis mimicked the extent of in vivo binding better than the other methods [24]. ED is also beneficial to drug binding research because of its reliability and cost effectiveness. The results obtained are under equilibrium conditions which are therefore representative of the true in vivo interactions [25]. This method requires the use of a membrane that is permeable to the drug but will retain the protein [25]. Known concentration and volume of drug are placed into one side of the well followed by a known concentration and volume of protein placed into the

same well. The other side of the well contains a non-interacting solvent such as universal buffer. As the drug diffuses across the membrane, some drug will bind to the protein while some will remain free and unbound. This diffusion continues until equilibrium is reached. Once equilibrium has been reached, the free concentration of drug can be determined allowing the identification of protein binding characteristics [25]. Figure 4 illustrates the general concept of equilibrium dialysis.

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Figure 5: Equilibrium across a semi-permeable membrane using the method of equilibrium dialysis [25] There has been much work done previously on bupivacaine's protein binding using ED, however most procedures did not use UV-spectroscopy to obtain the quantitative results. A study by Stumpe M et al. used ED to separate free and bound bupivacaine, followed by solid phase extraction and liquid chromatography with mass spectroscopy to determine the free bupivacaine concentration [26]. They did not use UV-spectroscopy in their analysis of bupivacaine. In terms of bupivacaine, the use of these two methods together is lacking in the literature and will therefore be used in this study to further understand the binding characteristics of this clinically relevant local anaesthetic.

Fluorescence Spectroscopy

Fluorescence spectroscopy like ED is a technique that has proven very effective in determining binding properties of drugs to proteins. This method consists of exciting molecules through irradiation at a particular wavelength (excitation) and measuring the radiation they emit at a different wavelength

(emission) [27]. This highly specific and highly sensitive method can be attributed to the fact that individual fluorophores excite and emit at specific wavelengths [27]. The tryptophan residues on BSA which are capable of fluorescing are responsible for detecting a fluorescence spectrum when bound to a non-fluorescent drug. This technique will be conducted in this project to confirm the binding of bupivacaine to plasma proteins. This will allow for a valid comparison between results that have been reported in other fluorescence spectroscopy studies as well as results obtained through ED. Lastly, understanding the specific intermolecular interactions that govern the binding of drugs to proteins such as hydrogen bonding, electrostatic bonding, Van der Waals forces and hydrophobic bonding are crucial in determining pharmacokinetic properties of new lead compounds. If binding is confirmed, fluorescence spectroscopy will also be used to determine the specific or non-specific interaction between bupivacaine and the test proteins.

Aims and Objectives

Aims

Firstly, the aim of this project is to determine if there is any protein binding between bupivacaine and BSA/AAG, and if so, the binding parameters of the interactions. Next, molecular forces responsible for this binding will also be determined. The next aim will be to investigate how proteins affect the binding and availability of bupivacaine to diffuse across a semi-permeable membrane. Lastly, this project will aim to determine if equilibrium dialysis and UV spectroscopy used together to quantify results will provide results comparable to accepted literature studies of bupivacaine.

Objectives

In order to achieve the above aims, the following objectives must be set out and accomplished. Obtain a UV-visible spectrum of bupivacaine dissolved in universal buffer. This provides an appropriate wavelength to set the Cecil spectrophotometer for future absorbance readings. Create a calibration curve of bupivacaine dissolved in universal buffer. Use fluorescence spectroscopy to determine any binding between bupivacaine and BSA at different drug concentrations. Determine the binding parameters from fluorescence spectroscopy results. Evaluate the major molecular interactions between bupivacaine and the two test proteins using sodium chloride in two concentrations. Determine the time taken for bupivacaine to reach equilibrium in the absence of BSA using equilibrium dialysis. Alter the BSA concentration to investigate the effect it has on the protein binding of bupivacaine using equilibrium dialysis.

Hypothesis

As stated above, the two most important human plasma proteins involved in bupivacaine's binding are AAG and HSA [18]. It is also stated that bupivacaine binds to AAG with higher affinity than HSA [18]. Therefore we can predict that binding with AAG and BSA will be observed and that the interaction with AAG will be stronger than that of BSA. Based on protein saturation kinetics, I predict that increasing the concentration of bupivacaine will increase drug-protein binding until all available binding sites are saturated. Once this saturation is observed, further addition of bupivacaine will not alter the binding. With the equilibrium dialysis experiment, I hypothesize that increasing the BSA concentration will cause greater drug-

protein binding leaving less unbound bupivacaine available to penetrate the semi-permeable membrane. Lastly, the addition of neutral sodium chloride should suppress the binding observed either fully or partially depending on the intermolecular forces that govern the binding.

Methods

Materials and Instruments

The Cecil Spectrophotometer (CE 1021 1000 Series) Carly Eclipse Fluorescence Spectrophotometer Spatula Electronic Weighing balance Electronic shaker Weighing boats Glass Beakers (50mL, 100mL and 200mL) Medical wipes Sonicator (XUBA 3) Multitwell pipette Graduated pipettes (20mL, 10mL) Pipette filler Gilson pipettes (100-1000 μ L / 2-20 μ L / 20-200 μ L / 2 μ L) Pasteur pipettes Yellow pipette tips Blue pipette tips Scintillation vials Glass quartz 4-sided cuvettes 1 mL acrylic cuvettes (Lot: 2375997) 96-well Micro-Equilibrium Dialysis Device (HTD9C) Adhesive cover for ED wells Methyl cellulose membrane for ED (MCWO 12-14 kDa / Batch number: 2132) The Standard operating procedures (SOPs) for the correct use of the above instrumentation are outlined in the appendix.

Chemicals

Bupivacaine hydrochloride (obtained from Sigma-Aldrich. Product number: B5274) Universal buffer composed of 10mM Citric Acid, 10mM Sodium DiHydrogenPhosphate, 10mM Boric acid (formulated by Karen Purcell and Mike Harrison) Bovine Serum Albumin (obtained from Sigma-Aldrich. Product number: 120M1919V) Alpha -1-Acid Glycoprotein (obtained from Sigma-Aldrich. Product number: 049k7565V) Distilled water Ethanol Dimethyl

sulfoxide, DMSO (obtained from Sigma-Aldrich. Product number: STBC8522V)Hydrochloric acid10% Decon solution (formulated by Karen Purcell and Mike Harrison)Sodium Chloride (NaCl) (Batch number: 837)

Cleaning Cuvettes

The strict SOP's in regards to cleaning cuvettes were followed throughout the duration of this project. 1mL glass 4-sided quartz cuvettes were used for the fluorescence spectroscopy experiments and 1mL acrylic cuvettes were used to obtain absorbance readings in the ED experiments. In order to avoid contamination that would disrupt or skew results, SOP 19 in the appendix was followed.

UV-Visible Spectrum of bupivacaine

The UV- visible spectrum of bupivacaine had previously been identified prior to the start of this project. This spectrum is given in both the water phase and the octanol phase. The spectrum in the water phase is the one of interest for this project and this wavelength is 271nm.

Calibration Curve

A Calibration curve was constructed in order to determine unknown concentrations of bupivacaine dissolved in buffer. This is done by comparing the unknown sample concentration to a set of known concentrations based on their absorbance values obtained through UV-spectroscopy. The Cecil spectrophotometer was used to obtain absorbance readings of known concentrations of bupivacaine dissolved in buffer. The spectrophotometer was set to a wavelength of 271 nm when analysing solutions of bupivacaine dissolved in buffer. The correct use of the spectrophotometer that was

undertaken is outlined in the appendix. The calibration curve produces a linear relationship between drug concentration and absorbance. This linearity indicates that as the concentration of drug doubles, the absorbance doubles as well. This calibration curve derives an equation that is used throughout the project to determine unknown drug concentrations from experimental absorbance readings. The reliability and confidence of this calibration curve is denoted as the R² value. The closer the R² value is to 1, the more reliable future readings from the calibration curve will be.

Calibration Curve for Bupivacaine in Universal Buffer

A 1 mg/mL solution of bupivacaine dissolved in pH 7.4 universal buffer was produced. First, a scintillation vial was 'tared' on an electronic balance. Then 14.4 mg of bupivacaine was added to the vial using a spatula. Next, to this scintillation vial, 14 mL of universal buffer was added using a 20 mL graduated pipette as outlined in SOP 8. A 1000 µL Gilson pipette was used to add a further 400 µL of universal buffer to the vial to ensure accuracy. The lid was then placed on the vial and shaken well by hand until the bupivacaine had fully dissolved in the buffer. Next, the Cecil spectrophotometer was used to obtain absorbance readings. In order to obtain appropriate readings for bupivacaine, the wavelength was set to 271nm. The spectrophotometer was then calibrated using a reference solution. This calibration was done by placing 1 mL of universal buffer with a Pasteur pipette to an acrylic cuvette and 'zeroing' the machine as outlined in SOP 13. Next, 1 mL of the 1mg/mL solution of bupivacaine in buffer previously prepared was added to a clean quartz cuvette using a 1000 µL Gilson pipette. The absorbance reading was recorded at a value less than 1.

Absorbance readings greater than 1 are inaccurate and indicate that the solution is too concentrated. Since this initial reading was below 1, serial dilutions were made to the 1mg/ mL solution and their absorbance readings recorded. Table : Serial Dilutions of bupivacaine dissolved in universal buffer

Concentration of bupivacaine in universal buffer (mg/mL)

Serial Dilution

14. 4mg of bupivacaine + 14. 4mL of universal buffer
0. 510mL of 1mg/mL bupivacaine solution + 10mL of universal buffer
0. 2510mL of 0. 5mg/mL bupivacaine solution + 10mL of universal buffer
0. 12510mL of 0. 25mg/mL bupivacaine solution + 10mL of universal buffer
0. 062510mL of 0.

125mg/mL bupivacaine solution + 10mL of universal buffer
00mL of bupivacaine solution + 20mL of universal buffer. From these known concentrations, absorbance readings were recorded. The data was then placed into Microsoft Excel and a graph was produced. This experiment was repeated a total of three times to ensure reliability. The mean results from the three trials were calculated and used to construct the final calibration curve which was used throughout the duration of this project.

Fluorescence Spectroscopy

The method of fluorescence spectroscopy was used to determine any binding between bupivacaine and BSA as well as AAG. This method was also used to distinguish the specific or non-specific molecular interactions that are responsible for this binding. The fluorimeter used throughout the project to obtain excitation and emission readings was the Carly Eclipse fluorescence spectrophotometer. Prior to obtaining intensity readings,

fluorimeter parameters had to be set. The scan was conducted from the range of 290nm to 900nm. Both the excitation and emission slit widths were set to 5nm. The fluorimeter sensitivity was set to high and the scan speed was set to fast. To determine if bupivacaine fluoresces alone, a control experiment was conducted where 50 μL of 100mM bupivacaine in DMSO solution was added to 950 μL of buffer. The fluorescence was recorded.

Stock Solutions of Bupivacaine, BSA and AAG

Once the parameters had been set, stock solutions of bupivacaine and BSA were produced. A 10mL solution of 100mM bupivacaine in dimethyl sulfoxide (DMSO) was produced. This was done by dissolving 0.3429g of bupivacaine in 10mL of DMSO. A 1mL stock solution of 200 μM BSA in universal buffer was then produced. This was done by dissolving 0.0133g of BSA in 1mL of universal buffer. Only slight shaking occurred as to avoid denaturation of the BSA. A 1mL stock solution of 200 μM AAG in universal buffer was also produced. This was done by dissolving 0.0074g of AAG in 1mL of buffer. Once again, the solution underwent slight shaking by hand until the AAG had completely dissolved.

The Effect of Protein Binding Using Fluorescence Spectroscopy

Binding between BSA and Bupivacaine

In order to calibrate the fluorimeter, 1000 μL of universal buffer using a 1000 μL Gilson pipette was added to a quartz cuvette and the emission spectrum recorded. This ensures that the buffer does not contain contaminants that interact with the BSA. To the same cuvette, 30 μL of

buffer was removed using a 200 μ L pipette and 30 μ L of 200 μ M BSA previously prepared was added using a 200 μ L Gilson pipette. A Teflon lid was then placed on the cuvette and inverted multiple times to ensure an evenly mixed solution before placing into the cuvette holder within the fluorimeter. The excitation spectrum is recorded at an emission wavelength of 280nm. To the same cuvette containing the BSA, incremental volumes (2 μ L) of 100mM bupivacaine previously prepared was added using a 2 μ L Gilson pipette and the emission spectrum was recorded. This was done until no further decrease in the fluorescence was observed. This experiment was repeated three times.

Binding between AAG and Bupivacaine

The above experiment was repeated with AAG in the same concentrations and manner.

Determination of DMSO's Role in Observed Binding

To determine if the DMSO that bupivacaine is dissolved in changes the chemical environment of the media and hence interferes with binding, a control experiment was conducted. This experiment was conducted in the same manner as the original fluorescence spectroscopy experiment described above, however instead of incremental volumes of drug being added to the cuvette, incremental volumes of DMSO was added.

Determination of Intermolecular interactions

In order to determine the specific or non-specific interactions that are responsible for the binding between bupivacaine and the two tested proteins, NaCl was used. Two concentrations of NaCl were used; 1M and 5M.

The 1M solution was made up by weighing 0.58g of NaCl powder and dissolving in 10mL of buffer. The 5M solution was made up by weighing 2.9g of NaCl powder and dissolving in 10 mL of buffer. The experiment described above in section 4.6.2 was then repeated in the same way except with the use of NaCl buffer solution instead of original buffer solution. Each concentration (1M and 5M of NaCl buffer solution) was tested. This experiment was conducted for both BSA and AAG.

Equilibrium Dialysis

Stock Solutions of Bupivacaine and BSA

Prior to use, dialysis membranes were soaked in universal buffer and 20% ethanol to prevent any contaminating growths. The membrane is then rinsed with universal buffer before being placed into the PTFE block by supervising technicians. Stock solutions of bupivacaine and BSA were then produced. A 2 mg/mL (5.83 mM) stock solution for bupivacaine was produced by weighing out 0.02g of bupivacaine using an electronic balance. This was added to a scintillation vial. To the same vial, 10mL of universal buffer was added using a 10mL graduated pipette and the lid placed on the vial. The vial was placed onto the electronic shaker until the bupivacaine had fully dissolved in the buffer. Two concentrations of BSA were produced in order to mimic the effects of low protein concentration and high protein concentration. For the low protein concentration, a stock solution containing 0.0385 g/mL (0.583 mM) was produced by weighing out 0.385g of BSA using an electronic balance. This was added to a scintillation vial followed by 10mL of buffer using a 10mL graduated pipette. The lid was placed on the vial and put on the electronic shaker until the BSA had fully dissolved in the buffer. For the

high protein concentration, a stock solution containing 0.385 g/mL (5.83 mM) was produced. This was done by weighing out 3.85g of BSA using an electronic balance to a 'zeroed' scintillation vial. To this vial, 10mL of buffer was added using a 10mL graduated pipette. The lid was placed on the vial and the vial was put on the electronic shaker until the BSA had fully dissolved in the buffer. Next, stock solutions of drug and protein that will be placed in the ED chambers must be produced. For the low protein concentration, this is completed by ensuring that 5.83 mM of bupivacaine is dissolved in 0.583 mM solution of BSA. In order to achieve this, 0.012g of bupivacaine is dissolved in 6mL of 0.583 mM BSA solution previously prepared. This new 6mL solution will be used in the ED chambers representing drug and low protein concentration. For the high protein concentration, in order to ensure that 5.83 mM of bupivacaine is dissolved in 5.83 mM of BSA, 0.012g of bupivacaine is weighed out and dissolved in 6mL of 5.83 mM BSA solution previously prepared. This 6mL solution will be used in the ED chambers representing drug and high protein concentration. These dilutions ensure that the concentration of bupivacaine in the sample side of each experiment is initially 2mg/mL.

Testing the Protein

In order to determine if the protein is retained by the semi-permeable membrane, an ED test was conducted. A solution of 5.83 mM BSA was produced the same way as described above. 150 μ L of this solution was placed into the sample side of three wells of the 96-well plate dialyser using a multiwell pipette. On the dialysate side of the same three wells, 150 μ L of universal buffer was added using a multiwell pipette. The adhesive cover was

placed on top of the wells and the dialyser was placed on the automatic shaker at medium level. Readings were taken from the dialysate side every hour for a total of three hours using the same method as described above.

Equilibrium Time of Bupivacaine

To determine the time taken for bupivacaine to reach equilibrium, 150 μL of the 5.83 mM stock solution of bupivacaine previously prepared was placed into the sample side. On the dialysate side, 150 μL of buffer was added. This was done for a total of 4 wells. Readings were taken from the dialysate side every hour for a total of 5.15 hours to determine the time taken to reach equilibrium. This experiment was repeated three times to allow for statistical analysis of the results. The absorbance readings that indicate equilibrium had been reached are outlined in the appendix.

The Effect of Protein Binding on Bupivacaine's Diffusion

A total of 12 wells were used for each experimental trial. 150 μL of universal buffer was added to the dialysate side of each of the 12 wells. For the control wells (BSA absent), 150 μL of the previously prepared 5.83 mM bupivacaine solution was placed in the sample side. This was done for the 4 wells using a multiwell pipette. The wells representing the drug and low protein concentration were filled next. This was done by placing 150 μL of the previously prepared stock solution containing 5.83 mM of bupivacaine and 0.583 mM of BSA into the sample side. This was done for 4 wells using a multiwell pipette. Next, the wells representing the drug and high protein concentration were filled. This was done by placing 150 μL of the previously prepared stock solution containing 5.83 mM of bupivacaine and 5.83 mM of

BSA into the sample side. This was done for 4 chambers using a multiwell pipette. After the 96-well plate dialyser had been filled with buffer and sample, an easily removable adhesive cover was placed on top of the wells in order to prevent evaporation, contamination and spillage during incubation. The 96-well plate dialyser was then placed on an automatic shaker at medium level in order to increase the rate of dialysis. ADD CIRCLE DIAGRAM ON PAINT HEREReadings were taken from the dialysate side every hour for a total of 5. 15 hours. In order to obtain absorbance readings, a 200 μL Gilson pipette is used to remove 100 μL from the dialysate side of one of each experimental well. This is then placed into a disposable acrylic 1mL cuvette containing 0. 9mL of buffer. This cuvette is placed into the previously calibrated Cecil spectrophotometer and the absorbance values are recorded. This experiment was repeated a total of three times in order to perform statistical analysis techniques to determine the experiment's reliability, accuracy and reproducibility.

Statistical Analysis

Great care and accuracy with all methods was taken to optimise results obtained during experimentation. In order to ensure that results are valid and reliable, experiments are repeated multiple times. Multiple readings portraying the same experimental variables and conditions allow for the use of statistical analysis to render the results significant or not. The statistical analytical tools used in this project are described below. Microsoft Excel 2010 was the program used in order to quantify and analyse statistical results. Most experiments in this project were repeated a minimum of three times to allow for statistical analysis. By repeating experiments multiple

times in consistent conditions, the mean was calculated which gives a more accurate description of what the result should be. The mean is calculated by taking the sum of the values and dividing by the number of times the specific experiment was done. This provides a single value which allows for simpler comparison between different sets of data. Determination of the mean also allowed for the calculation of the standard deviation and variance. These statistical components demonstrate to what extent values deviate from the mean. Values with low variance and standard deviations indicate that the value resembles that of the mean and hence is more accurate and reliable.

Correlation Coefficient

The correlation coefficient was calculated for the calibration curve of bupivacaine in buffer as previously stated. This statistical component describes the relationship between two variables [24]. Values for the correlation coefficient range from 0 to 1. The closer the R² value is to 1, the more reliable future readings from the calibration curve will be. An R² value of 1 indicates a perfect correlation.

T-Test

The t-test was used in this project to evaluate the differences in means between different sets of data. This test also provides information on the significance of the difference. This test denotes a p-value that indicates if there is a true difference between results. Therefore, a low p-value (<0.05) indicates the probability that there is a true difference between the data sets and this difference has not occurred due to chance or experimental/human

error. Since the comparison of data in this project is not matched pairs, an independent two-tailed t-test will be conducted.

Results and Discussion

UV-Visible Spectrum of Bupivacaine

In order to determine the concentrations of bupivacaine throughout the project, absorbance readings were taken using the Cecil spectrophotometer. From this previously determined UV-Visible spectrum, the peak absorbance is seen at 271nm. Therefore, when analysing solutions of bupivacaine in aqueous buffer, the spectrophotometer was set to 271nm. UV-Visible spectrum of bupivacaine in water with the peak absorbance seen at 271nm.

Calibration Curve for Bupivacaine in Universal Buffer

A calibration curve of bupivacaine dissolved in universal buffer was produced as outlined in section 4. 5. 1. To increase the accuracy and reliability of this calibration curve, the experiment was conducted three independent times. From the results of these three trials, a mean calibration curve was produced and used throughout the project to determine unknown bupivacaine concentrations in ED experiments. The results of the three trials are illustrated in figure ? along with the mean calibration curve illustrated in figure ? Calibration curve of bupivacaine dissolved in aqueous buffer for three separate trials measured at 271nm. Mean calibration curve of bupivacaine dissolved in aqueous buffer measured at 271nm illustrating standard error. The two calibration curves presented above indicate a linear relationship between bupivacaine concentration and the absorbance. As the concentration of bupivacaine increases, the absorbance increases as well.

The standard error was calculated for each data point and displayed as y error bars illustrated in figure ? As the concentration of bupivacaine increased, the standard error also increased. However, the standard error calculated was very low indicating that the variation between the three sets of data is minimal. The correlation coefficient was also calculated and shown in figure ? as an R² value of 0.9976. Since this value is close to 1, it suggests that the line of best fit is highly correlated to the trend of the plot and the equation produced ($y = 0.8297x$) is reliable to generate unknown bupivacaine concentrations in future absorbance readings.

Fluorescence Spectroscopy

A control experiment in the absence of BSA was conducted in order to determine if bupivacaine fluoresces alone. In order to do this, bupivacaine was added to DMSO and the emission spectrum was recorded as outlined in section 4.6.0. This experiment showed no fluorescence intensity indicating that bupivacaine does not fluoresce on its own. This allows for confidence in that any future fluorescence seen throughout this experiment is due to the protein and not bupivacaine.

The Effect of Protein Binding Using Fluorescence Spectroscopy

Binding between BSA and Bupivacaine

In order to ensure that the buffer does not contain contaminants that will interfere with the results obtained, the initial step was to record the intensity of buffer in the absence of drug and protein. The black line in Figure ? illustrates a negligible intensity reading which indicates that the buffer does

not interfere with the fluorescence results. Next, the excitation and emission spectrum of BSA was recorded using the parameters discussed in section 4.6.0. Figure ? illustrates the excitation spectrum at a wavelength of 280.16nm and an intensity of 670.23. The emission spectrum was recorded at a wavelength of 345nm and an intensity of 676.58. According to the literature, the two fluorescent tryptophan residues on BSA excite at a wavelength of 280 nm [28]. Therefore, the excitation wavelength of 280.16 nm that was obtained is consistent with the literature value. This experiment was repeated three times, however since the results were very similar, only the fluorescence spectrum of one trial will be evaluated and discussed. The results of the other two trials are found in the appendix.

Emission Spectrum

In

Buffer Alone

Excitation Spectrum

Caption: Graph showing the excitation (red line) and emission (brown line) spectrum of BSA. The emission spectrum of the buffer (black line) is also plotted. In order to confirm binding and extent of binding between bupivacaine and BSA, the emission spectrum was recorded. The concentration of 200 μ M BSA with incremental volumes of bupivacaine (100mM) dissolved in DMSO was used as outlined in section 4.6.2. If binding between BSA and bupivacaine occurs, the fluorescence intensity is expected to decrease with increasing concentration of drug until no further intensity decrease is observed. This is known as fluorescence quenching [29]. Figure ?

below depicts the emission spectrum obtained once a total of 28 μL of bupivacaine had been added incrementally. The major emission peak will be focused on as the peak in the middle may be due to the scattering of light. The minor peak may also be analysed to clarify any intensity recordings obtained. Intensity: 676.58 λ_{max} : 345nm Intensity: 428.76 λ_{max} : 336nm

Graph showing the fluorescence quenching spectra for 200 μM BSA dissolved in universal buffer with the incremental addition of 100mM bupivacaine. From figure ? it is clear that as the concentration of bupivacaine was increased the intrinsic fluorescence of BSA decreased. The initial intensity in the absence of bupivacaine was 676.58 and in the final reading dropped to 428.76 for an overall change in Intensity of 247.82. Analysing the shift in λ_{max} must also be taken into account as this can aid in the confirmation of binding between bupivacaine and BSA. From figure ?, the λ_{max} of BSA alone is seen at 345nm and the λ_{max} of the final reading is 336nm. This is a total shift of 9nm and is referred to as a blue-shift because it is a decrease in the wavelength [30]. The shift in λ_{max} may be attributed to conformational changes of the protein. BSA contains hydrophilic residues on its exterior and hydrophobic residues within its interior and is known to be very sensitive to its environment [30]. The maximum wavelength of BSA is seen when BSA is dissolved only in aqueous buffer where the tryptophan residues are exposed to the aqueous environment. According to Burstein et al. as the environment changes to a more hydrophobic one, blue-shifting is observed [29]. This is because the hydrophobic environment causes folding of the protein which causes the tryptophan residues to move into the interior of the protein where it is no longer as exposed to the aqueous environment

as it initially was [30]. This concept corresponds to the results obtained because as the basic bupivacaine was incrementally added to BSA, the environment of the test media became increasingly hydrophobic showing a total blue-shift of 9nm. Therefore, from the fluorescence quenching observed along with the change in λ_{max} , we can confirm that bupivacaine does interact with BSA to an extent.

Binding Parameters with BSA

Since binding between bupivacaine and BSA was confirmed above, binding parameters will be looked at next. Specifically, the dissociation constant (K_d) will be evaluated. For more accurate interpretation of fluorescence quenching data, the relative change of fluorescence will be used rather than whole emission intensity values. The formula used to accomplish this is $F_0 - F / F_0$ where F_0 and F represent the fluorescence intensities in the absence and the presence of bupivacaine respectively. The fluorescence intensity readings used are the means of the three trials. Graph illustrating the increase in relative fluorescence change as the concentration of bupivacaine increases measured at a wavelength of 345nm. From this graph, the general trend seems to be that as the concentration of bupivacaine increases, the quenching observed increases until saturation. This graph clearly distinguishes that this system has saturated indicating that all available binding sites on BSA have been fully occupied by bupivacaine. The plateau in figure? illustrates the saturation. It took approximately 2.153mM of bupivacaine for saturation to become evident. To obtain the K_d of this interaction a double logarithmic regression curve was produced as shown in figure?. This graph allows for the determination of the number of binding

sites and the binding constant for the interaction between bupivacaine and BSA. From this binding constant, the K_d can be calculated by taking the inverse of the binding constant. Figure?: Double logarithmic regression curve of bupivacaine concentration and relative change in fluorescence Equation: Double logarithmic regression curve [31] From Equation?, K represents the binding or association constant of bupivacaine with BSA, and n is the number of binding sites per protein molecule [31]. Q in this equation represents the quencher concentration which is bupivacaine. The binding constant is determined from the intercept and the number of binding sites is determined from the slope of the curve. From figure?, the linear equation produced is $y = 0.72421x - 0.532$. Table? Summarizes the results of the double logarithmic regression curve. Table?: the binding constant, the dissociation constant and the number of binding sites of bupivacaine with BSA.

Protein

K (mM)

K_d (mM)

n

R²

BSA 0.2943. 400. 7240. 9787 Therefore from this equation, the binding constant is 0.294 and the number of binding sites is approximately 0.7. The number of binding sites is approximately 1 indicating just one single binding site on BSA for bupivacaine. The correlation coefficient is 0.9787 which indicates the results obtained are highly correlated to the trend of the plot. From the binding constant, k_d is calculated to be 3.40mM. The calculated

dissociation constant of this interaction suggests weak binding between BSA and bupivacaine. The K_d concept suggests that the lower the K_d concentration, the stronger the binding/interaction. In this case, a K_d of 3.40mM is regarded as a very high concentration which indicates that a high concentration of bupivacaine is needed for half of the binding site on BSA to be occupied. Dissociation constants regarded as high affinity/strong interactions are typically in nanomolar or micromolar concentrations. The study conducted by Denson et. al suggests that the interaction observed between bupivacaine and albumin was low affinity binding [18]. Therefore, the calculated K_d from this regression curve is in accordance with accepted literature results.

Binding between AAG and Bupivacaine

Intensity: 273.16 λ_{max} : 334nm Intensity: 243.44 λ_{max} : 330nm Using the same parameters and concentrations for BSA as outlined in section 4.6.2.1, the binding between bupivacaine and AAG was evaluated. Due to time constraints, the experiment using AAG was only conducted once. Figure? below illustrates the fluorescence quenching spectra that was obtained for bupivacaine and AAG. Caption: Graph showing the fluorescence quenching spectra for 200 μ M AAG dissolved in universal buffer with the incremental addition of 100mM bupivacaine. From figure?, the initial intensity of AAG in the absence of bupivacaine was seen at 273.16 and a λ_{max} of 334nm. The final intensity after 28 μ L of bupivacaine had been added was recorded at 243.44 and a λ_{max} of 330nm for a difference in intensity of 29.72 and a blue-shift in λ_{max} of 4nm. When compared to the emission spectra of BSA and bupivacaine, it is evident that quenching was greater defined with the

BSA rather than the AAG. This indicates that the extent of binding with the BSA was much higher than the binding observed with the AAG. Figure ? graphically illustrates the relative change in fluorescence intensities as the concentration of bupivacaine increases for both the BSA and the AAG.

Caption: Comparison of the relative change in fluorescence of both AAG and BSA as the concentration of bupivacaine increases.

Binding Parameters with AAG

In order to compare the binding parameters of this interaction with BSA, a double logarithmic regression curve was also produced for this interaction. Figure? below illustrates the double logarithmic regression curve for the interaction between bupivacaine and AAG. Figure?: Double logarithmic regression curve of bupivacaine concentration and relative change in fluorescence. From this curve, the linear equation produced is $y = 0.5635x - 0.867$ and the correlation coefficient is 0.9495. However, this curve only represents the data points obtained before saturation. The reason for this is due to the inaccuracy of the regression curve when all the data points were plotted. When all of the data points were plotted including the saturation data, a correlation coefficient of 0.5684 was produced which is statistically unsatisfactory. Table ? gives the resulting values for both AAG and BSA. Table ? : The binding constant, the dissociation constant and the number of binding sites of bupivacaine with BSA and AAG.

Protein**K (mM)****Kd (mM)****n****R²**

BSA 0.2943. 400. 7240. 9787AAG 0.1367. 360. 5640. 9495

Table ? outlines the main findings and compares the two proteins used. From the Kd values in the table above, it is evident that AAG like BSA also shows weak binding/affinity but to an even greater extent. The number of binding sites found in AAG gave a value of 0.564 which like BSA does not exceed 1. Therefore with both proteins, it is assumed that only a single binding site is available for bupivacaine to bind. In the study by Denson et. al that was previously mentioned, the results of the interaction between bupivacaine and AAG was outlined as high affinity binding [18]. This indicates that the interaction between AAG and bupivacaine should show strong binding and should produce a much lower Kd than 7.36mM which was obtained from the regression curve in figure ? According to a fluorescence spectroscopy study conducted by Taheri et al on bupivacaine and AAG, the apparent Kd of the interaction at pH 7.4 is 1.85µM indicating that bupivacaine binds strongly to AAG [32]. From the two literature studies mentioned, it is clear that the interaction between bupivacaine and AAG is shown to have strong binding. However, the Kd obtained in this project is much larger than the literature value presented and could be attributed to a number of reasons. The dissociation constant presented in the literature is determined using derived equations for both neutral and protonated concentrations of bupivacaine.

The linear equation used in this project to determine the K_d may not have given an accurate description of the true K_d because this interaction may be more complicated and in turn does not follow the simple model of protein ligand binding seen in figure 4. Therefore the difference in equations used to determine the dissociation constants maybe responsible for the significant difference between the observed k_d and the literature k_d for this interaction.

Determination of DMSO's Role in Observed Binding

In order to confirm that the binding that was observed from the data above is only due to the interactions between the two proteins and bupivacaine, a control experiment was conducted as outlined in section 4. 6. 2. 3.

Bupivacaine is dissolved in DMSO to make up the stock solutions because this solvent aids in the complete dissolution of the drug. Because this solvent is being used, it is important to determine if DMSO changes the chemical environment of the media and in turn is responsible for any fluorescence quenching observed. The graph in figure? compares the difference in fluorescence intensities between incremental additions of DMSO and incremental additions of bupivacaine to BSA. The addition of DMSO to the BSA solution resulted in an overall decrease of 58. 8 in emission intensity and began to plateau following the third incremental addition. The addition of bupivacaine to the BSA solution resulted in an overall intensity change of 247. 82 as previously stated which clearly indicates binding is taking place. This control experiment concludes that the influence of DMSO on the binding observed is minimal and the alteration of the chemical environment of the media is negligible. Caption: Graph comparing the difference in fluorescence

intensities between incremental additions of DMSO and incremental additions of bupivacaine to BSA evaluated at a wavelength of 345nm.

Determination of Intermolecular interactions

Interaction between Bupivacaine and BSA

Once binding between bupivacaine and BSA was determined, the molecular interactions responsible for this binding were evaluated. In order to accomplish this, NaCl was added to the buffer solutions as outlined in section 4. 6. 3. Two concentrations of NaCl were tested; 1M and 5M. figure ? and ? illustrate the fluorescence results obtained from the 5M NaCl solution and the 1M NaCl solution respectively. Caption: Graph detailing the emission spectrum using 5M NaCl in the buffer solution measured at a wavelength of 345nm. Graph detailing the emission spectrum using 1M NaCl in the buffer solution measured at a wavelength of 345nm To compare these figures, a graph illustrating the relative change in fluorescence intensity and the concentration of drug added was produced. This graph compared the three buffer solutions used (buffer alone, buffer+ 1M NaCl, and buffer+ 5M NaCl). Caption: Graph comparing the relative fluorescence change of three solutions (buffer alone, buffer+ 1M NaCl, and buffer+ 5M NaCl) against corresponding bupivacaine concentration using BSA at a wavelength of 345nm. From figure? it is evident that binding is reduced significantly with the addition of NaCl. The buffer solution containing 1M NaCl experienced a total intensity drop of approximately 212 and a total shift in λ_{max} of 4nm. The buffer solution containing 5M NaCl experienced a total intensity drop of 193 and a total shift in λ_{max} of 3nm. When compared to the original fluorescence spectrum with buffer alone (intensity drop: 248 / λ_{max} shift:

9nm) it is obvious that the NaCl is suppressing the binding between bupivacaine and BSA.

Interaction between Bupivacaine and AAG

Similar to BSA, once binding between bupivacaine and AAG was observed, the molecular interactions responsible for this binding were evaluated. The same concentrations of NaCl (1M and 5M) were used in this experiment as outlined in section 4. 6. 3. Figure ? and Figure ? illustrate the results of this experiment. Caption: Graph detailing the emission spectrum using 1M NaCl in the buffer solution measured at a wavelength of 345nmCaption: Graph detailing the emission spectrum using 5M NaCl in the buffer solution measured at a wavelength of 345nmTo compare these figures, a graph illustrating the relative change in fluorescence intensity and the concentration of drug added was produced. This graph compared the three buffer solutions used (buffer alone, buffer+ 1M NaCl, and buffer+ 5M NaCl)Caption: Graph comparing the relative fluorescence change of three solutions (buffer alone, buffer+ 1M NaCl, and buffer+ 5M NaCl) against corresponding bupivacaine concentration using AAG at a wavelength of 345nmFrom figure?, it is evident that the NaCl also suppresses binding between bupivacaine and AAG. As seen with the BSA experiment, the addition of 5M NaCl to the buffer solution suppressed binding even more than the 1M NaCl solution. In both experiments this suppression is due to the large amount of sodium cations from the NaCl that shield the BSA/AAG and compete with the protonated bupivacaine for the protein binding site. The two different molar concentrations of NaCl were used in this experiment to determine if the 1M solution was sufficient to completely suppress binding or

if the addition of a higher concentration (5M) would have an effect. From figure? and figure ?, we can conclude that the addition of the 5M NaCl solution further suppressed binding compared to the 1M solution. This indicates that BSA and AAG had not been fully shielded with the Na⁺ cations and there is potential for further binding. From this experiment, we can furthermore conclude that electrostatic forces play a substantial role in the interaction between BSA/AAG and bupivacaine. However since binding is still being observed with both proteins, we can assume that there are other molecular forces such as, hydrophobic, van der Waals, or hydrogen bonds that are contributing to the observed binding. The study by Taheri et al which set out to understand the interaction between local anaesthetics and AAG determined that bupivacaine's binding to AAG is mainly mediated by electrostatic forces [32]. Therefore the results of this experiment are consistent with the literature and should contribute to the understanding of the binding sites of the two most important plasma proteins that are responsible for the binding of local anaesthetics like bupivacaine.

Equilibrium Dialysis

Testing the Protein

In order to ensure BSA is retained by the semi-permeable membrane in the ED wells, a control experiment was carried out as detailed in section 4. 7. 2. The three absorbance readings that were obtained from the dialysate sides after a total of three hours of dialysing were all 0. This indicates that no BSA had penetrated the membrane and provides confidence that future absorbance readings would only contain free concentrations of bupivacaine.

Due to time constraints, ED experiments were only conducted on BSA and not AAG.

Equilibrium Time

Initially, the time taken for bupivacaine to reach equilibrium was determined. To do this, a preliminary experiment was prepared as outlined in section 4. 7. 3. Readings were taken for a total of 5. 15 hours. The results of this experiment conclude that bupivacaine reached equilibrium at around 4. 15 hours. In the ED experiment of bupivacaine reported by Stumpe et al, equilibrium had been reached within 3 hours [26]. In this experiment, at approximately 3 hours, it is evident from table? that net transfer of bupivacaine had not yet been reached. The difference between the experimental time and the time reported in the literature may be due to a number of different reasons. The different bupivacaine concentrations used in either experiments or temperature differences between experiments or any other condition that might differ could all account for the difference in the time taken to reach equilibrium. All subsequent readings were taken at 4 hours to ensure equilibrium had been reached. Table?: The average absorbance readings with corresponding concentrations of three trials. The data highlighted in red represent the time taken for bupivacaine to reach equilibrium.

Well Number**Time (hrs)****Average Absorbance (Buffer)****Average Concentration (mg/mL)****1**

10. 0400. 048

2

3. 150. 0640. 077

3

4. 150. 0800. 096

4

5. 150. 0780. 094

The Effect of BSA on Bupivacaine Binding

Many trials throughout this experiment were attempted in order to obtain appropriate spectrophotometric absorbance readings at in vivo BSA and bupivacaine concentrations. When physiologically relevant concentrations were used, negligible absorbance values were obtained; hence it was decided to use concentrations that did not mimic in vivo conditions. This must be taken into account when comparing the results to the literature. To determine how proteins affect the diffusion of bupivacaine across a semi-permeable membrane, three experimental trials were conducted to observe binding as described in section 4. 7. 4. The first experiment was done in the absence of protein. The second experiment was done using a low

concentration of BSA (0.583 mM) while the third experiment observed the effects at a high concentration of BSA (5.83mM). The concentration of bupivacaine remained constant (5.83mM) in all three experiments. Each experiment was repeated three times to allow for statistical analysis. The table below gives the resulting data from the ED experiments. The experiment conducted with bupivacaine in the absence of BSA was used as a control. Note the initial concentration of bupivacaine in each well is 0.2mg/mL (5.83mM). All values represent equilibrium conditions. Table?: Table outlining the results of the ED experiments in the absence of BSA, low BSA concentration and high BSA concentration. This table shows that as the BSA concentration is increased, binding is also increased. Equation 3 was used to obtain percent binding.

Protein Concentration

Mean Bupivacaine concentration

Average % Binding

Standard Deviation

Standard Error

No protein

0.09700.00170.001

Low protein (0.583mM)

0.04577.50.00150.0008

High protein (5.83mM)

0.02189. 50.00260. 0015

From the results above, statistical analysis was performed and the resulting data is presented in figure?. T-Tests were conducted in order to determine if the change in protein concentration was significant. Comparison of no BSA to low BSA concentration yielded a p-value of 1.13×10^{-3} , and the comparison of no BSA to high BSA concentration yielded a p-value of 5.77×10^{-5} . These values indicate that the difference observed is very statistically significant. Lastly, comparing the low BSA concentration to high BSA concentration produced a p-value of 8.42×10^{-3} . With all three values being under the cut off range (p-value = 0.05), we can conclude that altering the BSA concentration has significant influence on bupivacaine's binding and the observed difference is a true statistical difference. From table?, the standard deviations are low indicating a close distribution about the mean. This indicates accuracy and reliability in the experimental work. The low standard error values indicate that the experimental results are close to the mean.

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figure? illustrates the fraction of bupivacaine unbound to BSA as calculated using equation 4. This shows that as the concentration of BSA increases, the amount of bupivacaine unbound decreases. These values represent the values of the three trials +/- Standard error. Statistical t-tests were performed against the control (No BSA) as well as the two samples containing BSA (* p < 0.05, **p < 0.001). As previously stated, the concentrations of bupivacaine and BSA used in this experiment were not representative of in vivo conditions. For this reason, directly comparing the

results to literature studies that used in vivo concentrations may not be possible. However comparing the general trends seen between this experiment and literature ED studies may be evaluated. Overall, as the concentration of BSA was increased, the binding seen also increased. With the low BSA concentration (0.583mM), the average binding of bupivacaine seen was 77.5%. As the BSA concentration increased to 5.83mM, the binding increased to 89.5%. A study that aimed to determine how disease-induced elevations of AAG influenced drug binding concluded results similar to mine [33]. This study reported that increases in plasma protein binding are due to increases in AAG concentrations which may influence the pharmacokinetic properties of the drugs [33]. Using equation 2, the K_d can be evaluated by taking the inverse of the K_a . For the low protein concentration (0.583mM), the calculated dissociation constant is 0.75mM. For the high protein concentration (5.83mM), the calculated dissociation constant is 6.5mM. As previously stated, dissociation constants in mM concentrations are regarded as being very high which indicates weak binding. However in this situation, because the protein concentration used is very high, equation 2 may not accurately calculate the K_d .

Conclusion

The overall results of this project conclude that protein binding does influence bupivacaine's ability to diffuse across a semi-permeable membrane. This was determined by achieving the objectives initially set out. Fluorescence spectroscopy confirmed that bupivacaine binds to both BSA and AAG which was expected, however the binding observed indicated weak interactions especially with AAG which did not support the hypothesis. These

weak interactions were concluded based on high K_d values obtained. The general trend seen with both proteins during fluorescence spectroscopy experiments were as the concentration of bupivacaine was increased, the drug-protein binding also increased until saturation. This supported the hypothesis. Equilibrium dialysis experiments indicated that bupivacaine was highly bound to BSA. As previously predicted, increasing the BSA concentration will cause an increase in binding thus leaving less unbound bupivacaine available to penetrate the semi-permeable membrane. This was the case as seen with the two different BSA concentrations used. Since physiologically relevant drug and protein concentrations yielded negligible UV-spectrophotometric absorbance readings, very high protein concentrations had to be used instead. This made it difficult to compare findings to literature studies and draw specific conclusions. The main reason for this is due to the fact that in order to read the sample in the UV-spectrophotometer, the sample had to be diluted in buffer in order to be read in the cuvette. This may explain why using UV-spectroscopy to quantify unknown drug concentrations has not been used in previous ED experiments. Lastly, electrostatic forces were concluded to be the main molecular forces responsible for the binding observed in this project.