

# [The drug binding proteins in the plasma biology essay](https://assignbuster.com/the-drug-binding-proteins-in-the-plasma-biology-essay/)

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Proteins play an important role in transporting endogenous and exogenous substances throughout the body. Such endogenous substances include bilirubin, vitamins and hormones.[1] [, #31@@hidden][Lindup, 1981 #31]Exogenous substances like most drugs will bind to proteins to a certain extent, and this drug-protein binding is important as it helps to distribute drugs throughout the body by transporting the drug via the blood stream to reach the site of action.[1] However, according to the general rule of ‘ free drug hypothesis’, for a drug to exert its pharmacological effect, it has to diffuse through physiological membranes as an unbound drug and interact with the effector site to produce a response.[1-4] It is assumed that unbound drug concentrations directly correlates with drug response.[4]Hence, the extent and affinity of a drug binding to plasma proteins is a major determinant factor in drug distribution, clearance and consequently, drug pharmacodynamics. It is therefore important that analytical methods to measure unbound drug and the drug-protein binding concepts are fully understood; enabling the quantification of drug-protein binding.[1]

## 1. 1. 1 Drug-binding proteins in the plasma

The human blood plasma contains over 60 different types of proteins such as albumin, lipoproteins and various other globulins.[5] These proteins are capable of binding to a wide variety of drugs, with sufficient binding affinity to elicit a significant effect on the drug’s pharmacological effect.[6] The two main drug-binding proteins in the plasma are albumin and α1-acid glycoprotein (AAG).[4, 6] The main focus of this project will be on these two proteins. Human Serum Albumin (HSA) being the most abundant in blood plasma is made out of 585 amino acids, with a molecular weight of 66-kDa.[5, 7] In healthy individuals, it is usually present at a concentration between 400 and 600uM.[6] HSA has multiple drug-binding sites, capable of binding drugs as well as endogenous ligands. There are two dominating binding sites that appear to have a greater binding affinity for acidic drugs.[1, 4, 6]AAG similarly has multiple drug-binding sites though it appears that only one site is dominant in drug binding, and it demonstrates a preference for basic and neutral drugs.[6, 8] It is a single stranded glycosylated amino acid made out of 204 resides, with a molecular weight of about 38 to 48-kDa. In a healthy individual, the plasma concentration of AAG ranges between 0. 55mg/ml to 1. 4 mg/ml, equivalent to about 12uM to 31uM. [6, 9-11] AAG is an acute-phase protein that is synthesized in the liver, hence in certain acute disease states such as liver disease or bronchitis, the concentration of AAG can decrease to plasma concentrations as low as 0. 02mg/ml. Whereas, in patients with acute myocardial infection or colon cancer, the plasma concentration can be as high as 3mg/ml (~60uM).[6, 10] Variations in drug binding caused by these disease states can affect both free and total drug concentrations.

## 1. 1. 2 Binding Concept

Assuming that the kinetics of association (k1) and dissociation (k-1) of the drug-protein complex is rapid, equilibrium between the unbound drug (D), the drug-protein complex (DP) and the protein (P) is quickly reached.[4, 12] The association and dissociation of drug-protein complexes can be illustrated using the following equilibrium schematic (Eq. 1).[4](1)At equilibrium, the rate of binding (k1[DP) is equal to the rate of dissociation (K-1[DP]). The drug binding affinity for its binding site on the protein can be expressed as the protein association constant (Ka, protein) (Eq. 2).[4](2)With reference to Eq. 3,[4] the extent of drug-protein binding can be expressed as the ratio of bound (Cbound) or free (Cfree) with the total (Ctotal) drug concentrations; and is presented as the percentage of drug bound to plasma protein (%PPB).[4](3)As plasma proteins may have one or more drug binding sites, the affinity and capacity of each binding site should be taken into account while calculating Cbound or Cfree.[4] As seen above, a drug’s pharmacological effect is significantly affected by drug-protein binding; which can also affects both pharmacokinetics and pharmacodynamics properties of a drug.[4]

## 1. 1. 3 Effects on Pharmacokinetics

The binding of a drug to plasma (and tissue) proteins is a major determining factor of drug disposition since only free drug can pass through membranes to produce a response at its target site.[5] Therefore, understanding the pharmacokinetics of a drug is crucial in establishing the relationship between the administered dose and drug plasma concentration, hence allowing suitable adjustment of doses to cater for individual patients.[14] Drug-protein binding has significant influence on two pharmacokinetic parameters, namely volume of distribution and elimination.[15]

## 1. 1. 3. 1 Volume of Distribution

Plasma proteins are found in blood plasma as well as interstitial fluids. The amount of albumin in interstitial fluid is comparable to that in plasma with respective volumes of 3 and 10 litres.[5]The volume of distribution (Vd) is a proportionality constant relating the amount of drug in the body to the drug concentration in the sampled body fluid (usually plasma).[4] The respective Vd obtained from blood plasma (Vd, plasma) may be calculated according to Eq 7.[4, 15, 16] Vp represents the volume of plasma, fuP and fuT indicates the fraction unbound in the plasma and the tissue respectively, CB represents the total concentration in plasma and V’T the volume in tissue, which includes the volume of blood cell and the extravascular space.[4, 16](4)Theoretically, with greater fuP, more drug is able to freely diffuse across the membranes, hence resulting in more extensive tissue distribution and a significant increase in Vd of total drug.[4, 5, 17] Ultimately, it is the ratio of fuP and fuT which determines the drug’s Vd. Since only the unbound drug is capable of leaving the capillaries, the rate of drug distribution into the tissues will be controlled by the unbound and unionized drug concentration gradient. Similarly, the rate of drug clearance can also be affected by unbound drug.[1]1. 1. 3. 2 ClearanceAny influence of plasma protein binding on drug elimination is best understood through clearance.[5] Clearance is defined as the volume of plasma from which a drug is completely removed per unit time, and is measured directly from the eliminating organ.[4] Most drugs are renally cleared (kidneys), while others are cleared via the liver. If a drug is cleared only by the kidneys, clearance can be illustrated from the following equation (Eq 8):(5)Since only the unbound drug is filtered, this indicates that protein binding is inversely proportionate to glomerulus filtration.[1, 3]Drugs that are cleared via the hepatic route, the following ‘ well-stirred’ equation (Eq 10) should be employed, assuming that the drug distribution in the liver is instantaneous and homogenous, and that the unbound drug concentration in the liver and in the blood leaving are equal:(6)Qhepatic represents the liver blood flow, fuB indicates the fraction unbound in blood, and CLuint represents the intrinsic clearance based on unbound drug concentrations. Protein binding can significantly affect drug clearance from the body especially if the drug is eliminated mainly via tubular secretion or hepatic metabolism, as high protein binding is associated with lowered drug elimination.

## 1. 1. 4 Effects on pharmacodynamics

It is often difficult to generalize the effects of protein binding on the Pharmacodynamics of a drug as there are many factors that need to be taken into consideration such as the drug mechanism of action, the drug-protein affinity, the protein concentration at the effect site and the location of the site of action.[18]

## The " drug free hypothesis" is based on two key concepts; firstly, the unbound drug concentrations on both sides of the membrane are equal at steady state; secondly, the free drug concentration at the site of action determines the pharmacological activity of the drug and the intensity of response.[19] Most drugs such as benzodiazepines and opiates demonstrate this hypothesis.[18, 20, 21]

## However, there are exceptions to this hypothesis as previous research has shown that for drugs such as A1 adenosine agonist, it is the total drug concentration that determines the response, instead of the free drug concentration.[18, 22]

Since drug response depends heavily on free, unbound drug concentrations at the site of action, physicochemical properties of the drug and transporters are vital components in dictating the extent to which the drug reaches its target.[4]

## 1. 1. 5 Protein binding measurement methods

To better understand the relationship between drug concentrations and pharmacological effects; the unbound fraction of the total drug concentration must be determined. There are a few methods to determine unbound drug concentration such as ultrafiltration, ultracentrifugation, equilibrium dialysis and gel filtration.[5, 23] In this study, equilibrium dialysis will be used.[24]

## 1. 1. 5. 1 Equilibrium Dialysis

Equilibrium dialysis is the most popular method and is generally accepted as the ‘ gold standard’.[6] It involves placing plasma sample on one side of the membrane and placing a drug-free buffer solution on the other side of the semi-permeable membrane.[5] The drug will diffuse through the membrane until an equilibrium is reached. By measuring the drug concentration on both sides of the membrane, the unbound drug fraction can be determined.[5]Figure 1: Illustration of Equilibrium Dialysis, taken from [51]This method requires variables such as assay temperature, type of dialysis membrane, drug concentration, ligand stability, plasma source and buffer (concentration, pH, composition) to be carefully controlled so as to avoid artifact.[6, 24]A number of studies used perspex cells for equilibrium dialysis,[9, 11, 25-27] while other studies used cellulose dialysis tubings soaked in distilled water.[28] In this experiment, the 96-Well Equilibrium Dialysis Block will be used. This apparatus is designed to address several limitations of currently available equilibrium dialysis equipment.[24] The unique property of this apparatus is the vertical placement of the dialysis membrane, which is sandwiched between two plastic blocks. This allows the dispensing and removal of the dialysate or sample without the hassle of having to disassemble the apparatus or terminating the assay during the experiment.[24] The block is also user friendly and easy to clean.[24]

## 1. 1. 6 Analytical methods

## The two main analytical methods that can be used to measure unbound drug concentrations are UV spectrometry and Spectrofluorometry.[25, 26, 29] Based on some literature, other methods that can be adopted are High Performance Liquid Chromatography (HPLC) and liquid scintillation counting.[9, 11]

## 1. 1. 6. 1 UV spectrometry

The UV absorbance spectroscopy is one of the oldest methods for determining drug concentrations, and it refers to absorption spectroscopy in the UV spectrum, and within this electromagnetic spectrum, molecules undergo electronic transitions from ground state to its excited state.[30]Figure 2: Illustration of UV absorbance using spectroscopy. Io indicates the intensity of incident radiation; It represents the intensity of transmitted radiationThe Beer-lambert law is a measurement of light absorption by a solution of molecules and is a linear relationship between absorbance and concentration of absorbing species.[30]This technique is complementary to fluorescence spectroscopy in that UV-spectrometry measures transitions from the ground state to the excited state, while a fluorometer measures from excited state to ground state.[30]

## 1. 1. 6. 2 Fluorescence spectrometry

The fluorescence spectroscopy uses a beam of light that is tailored for the type of fluorescence detection needed for the specific compound. The UV light emitted would excite the electrons within the molecule and cause them to produce an energy that is of lower energy than the energy absorbed.[30] The group of atoms responsible for the fluorescence process is known as fluorophores. Figure 3: Schematic diagram of fluorescence spectrophotometer, taken from [31]Generally, fluorescence is associated with rigid drug structures, such as phenols. Quinidine is an example of a fluorescent drug.[30] There is usually a linear relationship between the fluorescence emission and the analyte concentration, however, if the concentration of the analyte is too high, this proportion is no longer valid as unexcited molecules within the solution will reabsorb some of the fluorescence. For proteins, there are three fluorophores which enable them to exhibit fluorescence, they are tryptophan, tyrosine and phenylalanine.[32] Therefore by performing fluorescence on these drugs and proteins, qualitative information can be obtained regarding drug-protein binding affinity and capacity.

## 1. 2 Factors Influencing In-vitro Plasma Protein Binding

The measurement of plasma or serum levels of a drug is a widely accepted method to monitor and titrate clinical response although many drug serum concentrations and its clinical effects are not well correlated.[33]The four major factors that determine drug-protein binding are: protein concentration, drug concentration, drug-protein affinity and the presence of substances which might compete for the binding site and interfere with drug binding.[5, 27]During equilibrium dialysis experiment, there are several external factors that can alter drug-protein binding and they should be taken into consideration and carefully monitored. These factors include: equilibrium timing, temperature at which equilibrium took place and the pH of the protein-drug solution.[26]

## 1. 3 Quinidine Sulfate

1. 3. 1 Physical and Chemical PropertiesQuinidine is a dextrorotatory stereoisomer of Quinine and was originally derived from the bark of Rubiacae, a Cinchona species.[34] The alkaloid is available in several salt formations, namely, Quinidine Bisulfate, Quinidine Gluconate, Quinidine Polygalacturonate and Quinidine Sulfate.[34] Quinidine sulfate will be the focus of this report. Figure 4: Chemical Structure of Quinidine, taken from [34]Quinidine sulfate is an odourless, white or almost white, crystalline powder.[34, 35] It darkens on exposure to light and has a melting point of about 207°c.[34] The drug has a molecular weight of 783. 0; a pKa value of 4. 2 and 8. 8 and; a Log P (octanol/water) value of 3. 4, indicating its hydrophobicity.[24]With reference to Clarke’s Analysis of Drugs and Poisons, Quinidine is slightly soluble in water, with a solubility value of 1 in about 90 of water. Figure 5: Chemical Structure of Quinidine Sulfate, taken from [5]

## 1. 3. 2 Clinical Use

Quinidine Sulfate is an anti-arrhythmic drug that falls under class 1A of the Vaughn Williams classification and; it also exhibits anti-malarial schizonticide activities.[36] It is currently not licensed in the United Kingdom; however, it is widely used in the United States. It is mainly used for the treatment of arrhythmias by and to reduce the risks of recurrent atrial fibrillation after conversion to sinus rhythm. Occasionally, it is used to treat uncomplicated Plasmodium Falciparium malaria.[36] The usual daily dose of Quinidine sulfate is 600mg to 1600mg, depending on the indication, severity of the patient’s condition and other pharmacokinetic factors.[34, 36-38]

## 1. 3. 3 Pharmacology and Pharmacodynamics

Quinidine has been in the US market for a long time and its pharmacological properties are well-established.[38] Similar to other class 1 anti-arrhythmic drugs, Quinidine sulfate carries out its pharmacological effect by blocking the fast inward sodium current.[38] This decreases the inward sodium current in the cardiac muscle and Purkinje fibres, resulting in slowed conduction, prolonged effective refractory period and reduced automaticity within the heart.[38] Hence, preventing re-entrant arrhythmias and arrhythmias due to increased automaticity.[38]

## 1. 3. 4Pharmacokinetics

Quinidine Sulfate is an orally active drug, adhering closely to the Lipinski’s rule. It also has a high bioavailability of about 80%.[38] However, Quinidine Sulfate plasma levels might vary considerably between individuals after the administration of the same dose, however in healthy individuals, the relationship seems fairly constant.[39] Quinidine Sulfate has the following pharmacokinetic profile: Absorption: After oral administration, Quinidine sulfate is rapidly and almost completely absorbed due to its hydrophobicity.[34, 38]Distribution: Quinidine sulfate is rapidly and extensively distributed in most tissues, except the brain.[34, 38] It has a volume of distribution of 2 to 3L/kg in healthy young adults; but decreases (to as low as 0. 5L/kg) in patients with acute myocardial infarction.[34, 37, 38] The typical therapeutic drug plasma concentrations of Quinidine are 2 to 6 mg/L.[11, 34, 38]Metabolism: Quinidine undergoes first-pass metabolism in the liver and is mainly metabolized by cytochrome P450.[34, 37, 40]Elimination: Elimination occurs by catalyses of the parent drug into its metabolites via hepatic metabolism (60–85% of total clearance), followed by renal excretion of the remaining intact drug (15–40%).[37] Quinidine sulfate clearance is typically 3 to 5mL/min/kg in adults and usually twice or three times as rapid in children.[37]Woo and Greenblatt (1978) claimed that alterations in protein binding contribute importantly to variability in pharmacokinetic parameters and to patients’ clinical response to quinidine.[33]

## 1. 3. 5 Quinidine-Protein Binding Properties

A wide range of quinidine-protein binding percentages have been reported, however most values fall within the range of 65% to 90% in healthy adults.[40, 41] According to studies, the two main binding proteins for Quinidine are AAG and Albumin. Edwards et al. (1984) reported that in order to obtain a 20% increase in free plasma Quinidine, a 50% decrease in albumin concentration is required.[11]Hence, based on this finding, significant clinical changes in quinidine binding are more likely due to changes in AAG concentration, since serum levels of AAG may be increased in patients suffering from acute diseases such as acute myocardial infarction.[11]The four typical parameters that are important in understanding protein binding would be: drug therapeutic concentration (D), the dissociation constant (K), the number of binding sites (n) and the protein concentration (P). From these parameters, the binding affinity can be determined. According to Conn and Luchi (1960), their findings state that there is one receptor area for quinidine in each molecule of albumin (n= 1. 04). It is reported that the dissociation constant is 1. 3 x 10-4 at pH 7. 4.[46] Apparently, this is rather uncommon – one receptor area per albumin for quinidine to attach itself. Edwards et al. (1984) suggested that the human plasma had two main classes of binding sites that were identified as low-affinity high-capacity and high-affinity low-capacity binding site, with both sites contributing equally to the fraction of quinidine bound at therapeutic concentration.[11] It was also mentioned that a low-affinity, high-capacity binding site was found on albumin, while AAG was found to have a high-affinity, low-capacity binding site.[11]

## 1. 4Justification

As mentioned previously, Albumin and α1-acid glycoprotein have both demonstrated importance in quinidine binding. However, their relative roles in the serum binding of quinidine have been disputed, and their affinities have not been fully understood.[11] Therefore, the purpose of this work has been to investigate the variation in quinidine binding and affinity by varying albumin and AAG concentrations. This is important because changes in protein binding can have a profound effect on pharmacokinetic parameters such as clearance and volume of distribution.[26]

## 2. HYPOTHESIS

As mentioned previously, the two main binding proteins for Quinidine are AAG and Albumin,[42] and since quinidine sulfate is a basic drug, it should have a higher binding affinity to AAG as compared to albumin.[11, 41] Therefore, slight variations in AAG concentrations should significantly affect the bound drug fraction. Hence, this would mean that substantial clinical changes in Quinidine binding are more likely to happen due to changes in AAG concentration as compared to albumin, especially when serum AAG levels is elevated due to a number of common diseases, such as Acute Myocardial Infarction.[11]However, since this study involves the use of physiological concentrations of protein, therefore in spite of Quinidine Sulfate having a higher affinity to AAG, albumin would still be the major binding protein as it has a much higher plasma concentration as compared to AAG.

## 3. AIMS AND OBJECTIVES

Aims: To determine the binding characteristics of quinidine sulfate to two major plasma proteins, albumin and α1-acid glycoprotein; and how changes in these plasma protein concentrations might affect the bound drug fraction and consequently, its pharmacokinetics, especially after an Acute Myocardial Infarction.

## Objectives:

Identify which of these two methods, Spectrofluorometry or UV-spectrophotometry, would be more suitable in measuring physiological concentrations of quinidine sulfate. Determine the time taken for quinidine sulfate to diffuse across a semi-permeable membrane to reach equilibriumDetermine the binding affinities of quinidine sulfate with varying concentrations of BSA and α1-acid glycoproteinIdentify the effects of varying protein concentration on bound drug fractionTo qualitatively understand, based on the literature data and experimental results obtained, how an increase in α1-acid glycoproteins following an Acute Myocardial Infarction, would have an impact on the pharmacokinetics of quinidine sulfate

## 4. METHOD AND MATERIALS

## 4. 1 Apparatus

Refer to Table 1 for the list of apparatus used. The Standard Operating Procedure (SOP) for each apparatus is available in appendix 1. Apparatus1Perkin Elmer Lambda 12 UV/Visible Spectrophotometer2Cecil CE1021 Spectrophotometer3Shimadzu model RF-5301PC Spectrofluorophotometer4Synthetic quartz glass cuvette5Pasteur pipettes6Gilson micropipettes (100ul-1000ul, 20ul-200ul, 2ul)7Digital weighing scale8Glass Pipettes (1ml, 10ml, 20ml)9Pipette fillers10Yellow micro-pipette tips11Blue micro-pipette tips12Medical wipes13Weighing boats14Glass beakers (50ml, 100ml)15Eppendorf tubes16Metal spatulas17Scintillation vials18Electronic Shaker19Model HTD96b, High throughput 96-well dialysis block20Four-sided spectrofluorometry quartz glass cuvette21Disposable 1ml UV-cuvette22Dialysis adhesive sealing filmTable 1: List of apparatus used during experiment; refer to SOP in Appendix 1.

## 4. 2 Reagents

Reagents1Citric acid monohydrate, Sodium dihydrogen orthophosphate, Boric acid - Universal Buffer pH 7. 42Distilled water3Ethanol4Sigma-Aldrich Q0875-25G, Quinidine sulfate salt dihydrate5Sigma-Aldrich G9885-199MG, 1-acid Glycoprotein, from human plasma6Sigma-Aldrich A7906-100MG, Albumin, from bovine plasmaTable 2: List of reagents used during experiment; refer to SOP in Appendix 1.

## 4. 3 UV-Spectrum of Quinidine Sulfate in Universal Buffer

Perkin Elmer Lambda 12 UV/Visible Spectrophotometer was used to determine the UV-spectra wavelength of Quinidine Sulfate. A 0. 05mg/ml Quinidine Sulfate solution was prepared by dissolving 10mg of Quinidine Sulfate in 10ml of universal buffer pH7. 4, followed by a 20 times dilution. The drug was scanned between the wavelengths of 200nm to 600nm, and at a scan speed of 240nm/min with 3nm data interval.

## 4. 3. 1 UV-spectrophotometry Calibration Curve of Quinidine Sulfate

A calibration curve is essential in establishing the relationship between the UV-absorbance reading and the amount of drug present.[53] This usually involves the preparation of a set of standard solutions containing a known concentration of drug. The absorbance is then measured for each standard solution and from there, a relationship is established; enabling an estimation of unknown drug concentrations through the calibration curve.[53]The absorption spectra of Quinidine Sulfate were obtained using a Cecil CE1021 Spectrophotometer. The Spectrophotometer was set at a wavelength of 331nm and operated with reference to the ‘ University of Manchester: SOP 15’ (Refer to appendix 1). Three sets of five known concentrations (0. 01, 0. 005, 0. 0025, 0. 00125, 0. 000625mg/ml) of Quinidine Sulfate dissolve in Universal buffer pH7. 4 were prepared and measured in a synthetic quartz glass cuvette to obtain a calibration curve (refer to Appendix 3 for data).

## 4. 4 Fluorescence Emission Spectrum of Quinidine Sulfate

As Quinidine Sulfate is a fluorescent drug, Shimadzu Model RF-5301PC Spectrofluorometer was used to measure the intensity of emission from different concentrations of Quinidine. A 0. 1mM stock solution of Quinidine dissolved in Universal Buffer pH7. 4 was prepared. 2μl of the stock solution was diluted in 1ml of buffer and measured in a four-sided spectrofluorometry quartz glass cuvette. The wavelength at which quinidine had emitted was recorded.

## 4. 4. 1 Fluorescence Spectroscopy Calibration Curve of Quinidine Sulfate

A 0. 01mM stock solution of Quinidine Sulfate in Universal Buffer was prepared. Spectrofluorometer parameters were adjusted to λex= 331nm, with a slit width of 5nm. The emission spectra was measured between the range of 340nm to 600nm; for Quinidine Sulfate specifically, intensity measurements were taken at λem= 381nm. 1ml of Universal buffer was pipetted into a 1cm four-sided quartz glass cuvette and intensity readings were measured after each addition of stock solution at 2μl intervals. Three sets of ten concentrations were measured and recorded to obtain a calibration curve (refer to appendix 3 for data).

## 4. 5 Equilibrium Timing of Quinidine Sulfate

Obtaining the equilibrium time of Quinidine Sulfate is important to ensure that when equilibrium dialysis is performed, the measurement of the unbound drug taken from the dialysate is only measured when equilibrium has reached. Two 20ml stock solutions were prepared to determine the time taken to reach equilibrium in the absence and presence of BSA: 1. Stock solution (BSA): 530μM BSA and 639μM Quinidine sulfate in buffer2. Stock solution (drug only): 639μM Quinidine Sulfate in bufferEqual volumes (150μl) of stock solution and buffer were added on each side of the membrane to prevent osmotic pressure from interfering with the diffusion of the unbound drug. Dialysate (To be measured by spectrofluorometer) [150μl]Sample (From prepared sample solutions) [150μl]Membrane (Mimic physiological membrane barriers)Only unbound (fu) drug is able to diffuse cross the membrane barrierFigure 6: Illustration of unbound drug diffusing through membrane during equilibrium dialysisSample and dialysate drug concentrations obtained from the equilibrium dialysis were measured using a UV-spectrophotometer set at λ= 331nm. Three readings were collected for each sample and dialysate at one-hour intervals so that an average of the values can be obtained. Figure 7: Equilibrium Dialysis design to obtain Equilibrium timing for Quinidine Sulfate100μl of both the sample and dialysate was individually added into a disposable 1ml UV-cuvette and diluted 10 folds with universal buffer to allow measurements to be taken. The calibration curve obtained via the method mentioned in section 4. 3. 1 was used to calculate the free drug concentration in the dialysate and sample. To ensure that equilibrium has taken place, drug concentrations on both sides of the well should be equal.

## 4. 6 Equilibrium Dialysis of Quinidine Sulfate with Bovine Serum Albumin and α1-acid Glycoprotein using Two Drug and Protein Concentrations

As bovine serum albumin (BSA) has similar structural homology with human serum albumin (HSA), it was used in replacement of HSA in this experiment.[52] To simulate the physiological concentration of HSA in the plasma, two BSA concentrations (low and high) of BSA were used; they were 303μM and 606μM respectively. The spectrofluorometer was chosen to measure the unbound drug concentration, as it is a more sensitive instrument; allowing the measurements of very small amounts of drug. The drug concentration used in this experiment simulates the concentration of drug present in the physiological plasma of a patient. A low (6. 4μM) and high (19. 2μM) drug concentration was used. The following stock solutions were prepared and contained in scintillation vials (see table 3):

## Label

## Stock solution

## Volume

AUniversal Buffer20mlBAlpha1-acid glycoprotein, at 1. 5mg/ml10mlDBSA in buffer, at 80 mg/mL10mlC1Drug in buffer, at 0. 01 mg/mL10mlC2Drug in buffer, at 0. 03 mg/mL10mlTable 3: Stock solutions prepared for equilibrium dialysis, involving BSA and AAGSpecific volumes of the stock solutions were transferred into their respective labelled eppendorf tubes (refer to appendix 3 for individual solutions prepared). 150μl of Universal buffer was filled on the dialysate side of all the wells, while 150μl was removed from each eppendorf tube and placed into the sample side of the equilibrium dialysis well. Two more repeats were done for each eppendorf tube (see figure 8). Figure 8: 96-well Micro-Equilibrium dialysis device layout of Quinidine Sulfate for both BSA and AAGThe equilibrium dialysis device was placed on an electronic shaker for 5 hours. Once the equilibrium time has reached, the device is removed from the shaker. 100μl of dialysate was removed from individual wells and placed in a four-sided quartz glass cuvette where it was diluted 10 folds with buffer. The cuvette was placed in the spectrofluorometer, allowing the unbound drug concentration to be measured.

## 4. 7 Equlibrium Dialysis of Quinidine Sulfate with α1-acid Glycoprotein and Bovine Serum Albumin at a ratio of 1: 1

In order to understand the binding affinity of Quinidine Sulfate to the two different proteins, a ratio of drug to protein should be 1: 1. Therefore, low (6. 4μM) and high (19. 2μM) concentrations of Quinidine Sulfate and proteins were used. The following stock solutions were prepared (see table 5).

## Label

## Stock solution

## Volume

AUniversal Buffer20mlC1Drug in buffer, at 0. 01 mg/mL10mlC2Drug in buffer, at 0. 03 mg/mL10mlBBovine Serum Albumin, at 4. 224mg/ml10mlDAlpha1-acid glycoprotein, at 2. 624mg/ml10mlTable 4: Stock solutions prepared for equilibrium dialysis, involving AAG and BSASpecific volumes of stock solutions were mixed in eppendorf tubes making up to 1ml (refer to appendix 3 for individual eppendorf solutions prepared). 150ul of each solution was transferred into individual well. The equilibrium dialysis layout is the same as the layout in figure 8. For procedures on measuring the unbound drug concentration, refer to section 4. 6.

## 4. 8 Binding Affinity

## 4. 8. 1 Drug-protein bound fraction calculation

## Once the equilibrium dialysis is performed, a Shimadzu Model RF-5301PC Spectrofluorophotometer is used to measure the free drug concentration on the dialysate side of the wells. The drug concentration is determined from the intensity by using the calibration curve in section 4. 4. 1.

## The unbound drug fraction is calculated using the following equation (equation 7):

(7)fu represents the unbound fraction, while Cf represents the free drug concentration, C0 represents the original drug concentration. From there, the bound fraction can be calculated by subtracting the unbound drug fraction (refer to equation 8).(8)

## 4. 8. 2 Relationship between Quinidine Sulfate ratio binding to BSA and AAG

The extent of protein binding interaction was determined at equilibrium and was expressed as the ratio between molar concentrations of bound quinidine (B) and free unbound quinidine (F). B/F is plotted against protein molar concentration, P, to attain a linear relationship.(9)According to the law of mass equation above (equation 9), the gradient obtained from the graph would determine the binding affinity, nK. K represents the association constant of the binding site and n is the number of binding sites on a protein molecule available for drug binding. Linear regression and correlations was done to ensure the results were significant (refer to section 4. 10).

## 4. 9 Variation of Protein concentration and its implication on the pharmacokinetics of Quinidine Sulfate

A simplified mathematical model was used to calculate the affinity of quinidine to the two different proteins. It was also used to predict the effects of varying protein concentration on bound drug concentration. This was done using the nK parameter that was determined in section 4. 8. The macros of the mathematical model can be viewed in the appendix 3. The y-axis of the graph plotted was bound fraction, while the x-axis used was protein-to-drug ratio. The sigmoidal curve was plotted by varying the protein concentration; and at physiological drug concentrations, the position of the protein to quinidine ratio can be found within the graph.

## 4. 10 Statistical Analysis

The following analysis was performed using Excel Spreadsheet and Excel Data Analysis Toolpak.

## 4. 10. 1 Descriptive statistic

The average mean of a set of results were calculated to represent the central tendency of the data. It was calculated by dividing the sum of individual values with the number of values (n). However, the average mean alone is not a good representation of the data especially when there are outliers. The standard deviation (SD) was calculated as a measure of the spread of measurements. It is an estimate of the degree of data distribution and it helps to describe how individual data deviates from the sample mean.[43]Standard error is related to SD by estimating the probability of error between the calculated mean and the true mean. When calculating the standard error for a ratio, the following equation was used: . (10)Error bars were then added onto the graph.

## 4. 10. 2 Test for multiple measurement variables

Linear regression and correlation was used to explore the link between the bound fraction and protein-to-drug ratio by determining: the r2 value (co-efficient of determination) that describes the strength of the relationship between the variables, the P-value of the hypothesis test, and the regression line that illustrates the linear relationship.[43] In this study, linear regression was used to test the hypotheses on the cause of increasing protein concentration and its effect on bound drug fraction.[43]The test statistic for a linear regression would require the use of the Data Analysis Toolpak. Therefore, to establish a statistically significant relationship between the two variables, the p-value should be less than 0. 05. Strongly correlated values would have a p-value <0. 01.

## 5. RESULTS AND DISCUSSION

## 5. 1 UV-Spectrum of Quinidine Sulfate

Figure 9: The UV-spectrum of Quinidine Sulfate in Universal Buffer pH7. 4; peaks at 281nm and 331nmBSA has an absorbance peak of 280nm[44, 47, 48, 49, 50], which is very close to the first peak of Quinidine sulfate (281nm) demonstrated in figure 9, hence 331nm was chosen as the wavelength for Quinidine Sulfate in this experiment to avoid any interference which might be caused by the presence of BSA.

## 5. 2 Fluorescence Emission and Excitation Spectrum of Quinidine Sulfate

## Emission

ExcitationThe following figure 10 demonstrates that Quinidine Sulfate is a fluorescent drug. This could be due to the structure of Quinidine Sulfate as it has several fluorophores. Figure 10 indicates that Quinidine sulfate excites at a wavelength of 331nm and emits at a wavelength of 381nm. Figure 10: The Excitation and Emission wavelength of Quinidine Sulfate; λex= 331nm and λem= 381nm respectively

## 5. 3 Calibration Curves of Quinidine Sulfate

## The following graphs, figure 11 and 12 show the calibration curve of quinidine sulfate. The graphs were plotted against known concentrations of quinidine dissolved in universal buffer. Three readings were attained for each concentration, and the mean was calculated to ensure accuracy. A best-fit line intercepting through the origin was plotted to indicate a linear correlation between absorbance and drug concentration, with both graphs having a regression value (R2) of > 0. 99.

## Standard error was calculated for each data point to represent the standard deviation from the mean. The gradient obtained from each graph will be used to determine the corresponding unbound quinidine sulfate concentrations in later experiments, allowing the calculation of bound drug fraction (fb).

## 5. 3. 1 UV-spectrophotometry Calibration Curve

The graph in figure 11 was plotted against known concentrations of quinidine sulfate and the absorbance was measured at a wavelength of 331nm. Refer to section 4. 3. 1 for detailed procedures. Figure 11: Calibration curve of Quinidine Sulfate using a spectrophotometer (uM)

## 5. 3. 2 Fluorescence Spectroscopy Calibration Curve of Quinidine Sulfate

Figure 12 is a graph showing spectrofluorometry intensity plotted against known concentrations of quinidine sulfate, with the emission intensity of each sample measured at a wavelength of 381nm. Refer to section 4. 3. 2 on the detailed procedures. Figure 12: Calibration Curve of Quinidine Sulfate using a spectrofluorometer

## 5. 4 Quinidine Sulfate Equilibrium Time

As there are variations in the information regarding the equilibrium dialysis timing of quinidine sulphate; this experiment was done to determine the time needed for equilibrium to take place. The apparent free drug concentrations on both sides of the well were compared using equilibrium dialysis in universal buffer. The absorbance of quinidine sulfate was measured on both sides of the dialysis well (dialysate and sample) at each hour using a UV-spectrophotometer. Figure 13: Absorbance of quinidine sulfate at every hour (reflection of the concentration); time taken for quinidine sulfate to equilibrate through a semi-permeable membrane in equilibrium dialysis determines the equilibrium time, in the absence of protein. According to previous studies, equilibrium is usually achieved within 6 hours[5], though some research papers have claimed to allow 18 hours of equilibrium time.[9] However, as seen from the graph in Figure 13, the plateauing of absorbance indicates the time taken to reach equilibrium. Hence, this shows that the results agree with literature and that 4 hours is sufficient for Quinidine Sulfate to achieve equilibrium on both sides of the dialysis well when no protein was involved.[26]By keeping the experiment conditions the same, the equilibrium timing experiment was repeated, but this time with the addition of BSA. The temperature, buffer and quinidine concentration were kept the same. Figure 14: Absorbance of quinidine sulfate at every hour (reflection of quinidine concentration); time taken for quinidine to equilibrate through a semi-permeable membrane determines the equilibrium time, in the presence of BSAAs seen from the graph in Figure 14, the time taken for Quinidine Sulfate to reach equilibrium in the absence or presence of protein is also around 4 hours. However, at the 4th hour, it seemed that even though the sample side of the well had remained constant after the 3rd hour, there was still a slight increase in absorbance on the dialysate side of the well. This could be partly due to the presence of protein, which could have prolonged the time taken to reach equilibrium as compared to that in the absence of protein. It could also have been due to experimental errors during the dilution of the samples before measuring. Nonetheless, to ensure that equilibrium has taken place, equilibrium dialysis should be ran for 5 hours. Apart from the equilibrium timing, this experiment corroborates with other literature demonstrating that quinidine binds to albumin.[41] It also shows that equilibrium dialysis is suitable for quinidine sulfate and that equilibrium of quinidine takes place without having any unexpected interaction with the semi-permeable membrane. However, this result cannot confirm that BSA does not interfere with the absorbance reading of quinidine when measuring the sample.

## 5. 5 Calculation of Bound Fraction from Equilibrium Dialysis

Wells from the equilibrium dialysis were measured using the spectrofluorometer instead of the UV-spectrophotometer because minute amounts of quinidine were used to simulate the physiological plasma concentration of quinidine; therefore, only a sensitive instrument as such is suitable to detect such small amounts.

## Drug Conc. (uM)

## 6. 4

## 19. 2

## Fraction of Unbound drug (fu)

## Fraction of Bound drug (fb)

## Fraction of Unbound drug (fu)

## Fraction of Bound drug (fb)

## Protein conc. of BSA (uM)

## Control

0. 000. 000. 000. 00

## 0

1. 000. 001. 000. 00

## 303

0. 710. 290. 570. 43

## 606

0. 490. 510. 440. 56

## 6. 4

1. 000. 001. 000. 00

## 19. 2

1. 000. 000. 940. 06

## Protein conc. of AAG (uM)

## Control

0. 000. 000. 000. 00

## 0

1. 000. 001. 000. 00

## 3. 7

0. 890. 110. 850. 15

## 18. 3

0. 690. 310. 740. 26

## 6. 4

0. 920. 080. 880. 12

## 19. 2

0. 600. 400. 650. 35Based on the results obtained from the equilibrium dialysis, the following bound and unbound drug fraction can be calculated. Table 5: The bound and unbound drug fraction obtained from equilibrium dialysis. Values highlighted in purple indicate the bound drug fraction at a protein-drug ratio of 1: 1; Data highlighted in green indicate the physiological protein concentration in plasma; Data highlighted in red indicate the physiological protein concentration used to obtain a protein-drug ratio of 1: 1. With reference to table 5, the controls (equlibrium dialysis with protein and buffer only) used in the experiment had zero unbound fraction, hence indicating that protein could not pass the semi-permeable membrane and interfere with the intensity measured. It also meant that there was no drug or other contaminants present which could potentially disrupt the experimental results and create a false positive result. In addition, the equlibrium dialysis ran with zero protein concentration (quinidine and buffer only) had zero bound fraction, indicating that the results obtained were valid and the equilibration took place without interacting with the dialysis membrane to create any false positive or negative results. Figure 15: Bound fraction of quinidine versus varied BSA concentrationsFigure 16: Bound fraction of quinidine versus varied AAG concentrationsBased on the results obtained from the equal drug and protein concentration ratio (refer to table 5) at a quinidine concentration of 6. 4uM, no drug was bound to BSA in comparison with 0. 08 in AAG. Similarly, at a 1: 1 quinidine and protein concentration of 19. 2uM, BSA had 0. 06 of drug bound in comparison with AAG, which had a considerably higher drug-binding fraction of 0. 4. This would suggest that quinidine sulfate has a higher binding affinity to AAG as compared to BSA. At physiological conditions, the amount of albumin in the plasma ranged between 15 to 100 times more than AAG, however, the bound fraction difference was not proportionate; with BSA being only slightly two times more than AAG. This data supports my hypothesis, because when equal number of moles of BSA was added to quinidine, little binding occurred. However, when the same number of moles of AAG was added, there was a substantial amount of binding that took place. However, based on figure 16, it seemed that the points were not as well correlated as compared to BSA, and the bound fraction calculated for 6. 4uM and 19. 2uM drug concentration at 3. 66uM AAG concentration was an outlier, being higher than expected. This error could have been a result of experimental errors due to inaccurate dilutions when making up stock solutions; as minute concentrations of AAG and quinidine were used, hence hundred times of dilutions had to be made.

## 5. 6 Relationship between protein concentration and bound drug concentration

The protein concentration used in the experiment ranged between normal plasma serum concentrations to concentrations that demonstrated one to one protein to drug ratio. The binding ratio (B/F) ranged from 0. 00 to 1. 40 for BSA and 0. 00 to 0. 80 for AAG, with B representing the bound molar drug concentration, while F represented the free unbound molar drug concentration. The actual BSA and AAG concentrations used in the equilibrium dialysis and the B/F ratio are shown in table 6. The binding ratio (B/F), were plotted against the corresponding concentrations of AAG and BSA in figure 17 and figure 18 respectively.

## B/F ratio

## BSA concentration (uM)

## AAG concentration (uM)

## 6. 4

## 19. 2

## 303

## 606

## 3. 66

## 6. 4

## 18. 3

## 19. 2

## Drug concentration (uM)

## 6. 4

0. 000. 000. 391. 020. 120. 080. 450. 65

## 19. 2

0. 000. 060. 761. 290. 170. 140. 350. 53Table 6: Binding ratio of quinidine under different concentrations of AAG and BSA used in equilibrium dialysisFigure 17: Relation of concentrations of BSA to the binding ratios (Bound/Free) of Quinidine Sulfate determined by equilibrium dialysis. The higher and lower dashed lines represent the best-fit line for high and low drug respectively. The solid linear line is the best fit line for all data points (high and low drug concentration); determined via least squares regression analysis (r2= 0. 95, P <0. 001). Gradient of best-fit line represents the nK value. Error bars represent standard error. Figure 18: Relation of concentrations of AAG to the binding ratios (Bound/Free) of Quinidine Sulfate determined by equilibrium dialysis. The higher and lower dashed lines represent the best-fit line for high and low drug respectively. The solid linear line represents the best-fit line for all data points (high and low drug concentration); determined via least squares regression analysis (r2= 0. 817, P <0. 01). Gradient of best-fit line represents the nK value. Error bars represent standard error.

## R2-value

## P value

## nK value (uM-1)

## Standard error

## Lower 95%

## Upper 95%

## Albumin (BSA)

0. 9500. 000040. 001920. 00180. 001480. 00236

## α1-acid Glycoprotein (AAG)

0. 8170. 002100. 026620. 00520. 014040. 03919Table 7: The linear regression and correlation values of figure 17 and figure 18. Refer to Appendix III. The linear correlation of bound-to-free drug ratio and BSA concentrations is strongly significant (R2= 0. 95, P <0. 001); the linear regression line is shown in figure 17. AAG seems to bind quinidine extensively and the bound-to-free drug ratio and AAG concentration is also significantly (R2= 0. 82, P <0. 01) correlated to the concentration of protein. Table 7 shows that there is a strong linear regression and correlation between protein concentration and B/F ratio, hence demonstrating that with increasing protein concentrations, there would be an increase in bound drug concentration. Based on the experimental results, there is a strong linear correlation between the drug binding ratio and albumin, however, it was reported by Nilson (1977) that there was no significant correlation observed between them.[9] This discrepancy in results could be explained by the difference in albumin concentration used. The presence of a significant correlation could be due to the large albumin concentration range used in this study; whereby very low (6. 4uM) and high (606uM) concentrations of albumin were used. Whereas, in Nilson’s study, only physiological serum concentrations of albumin had been used, which was a rather narrow range of concentrations which did not vary much.[9]With reference to the results, AAG had a weaker linear correlation (R2= 0. 82, P <0. 01) as compared to BSA probably due to the influence of a narrow AAG concentration range which had been used in this study. From the gradient of the two curves, the binding affinity, nK, of Quinidine Sulfate to BSA is 1. 9x10-3 uM-1, while the binding affinity of AAG is 2. 66x10-2 uM-1. This again supports the hypothesis that the affinity of quinidine with AAG is greater than that of BSA. This is probably due to the basic nature of the drug; as a number of studies have shown that basic drugs tend to have higher affinity to AAG and is less affected by albumin variations. [11, 41]Therefore, due to quinidine’s higher affinity with AAG, this may lead to the assumption that: patients with increased AAG plasma concentrations due to an acute myocardial infarction[42, 45], would have a significant increase in drug-protein binding. The effect of protein concentration variation on drug binding and its affinity will be further explained in section 5. 7.

## 5. 7 Variation of Protein concentration and its implication on the pharmacokinetics of Quinidine Sulfate

The drug-protein binding is characterised by the parameter, fraction unbound (fu) or fraction bound (fb). A simplified mathematical model was used to understand the results obtained, and to investigate how bound drug fraction varies with protein concentration, and subsequently understand how these protein variations might affect the pharmacokinetics of quinidine. As mentioned, this model is a simplified model, therefore this model is used under the assumption that the kinetics of binding and dissociation are rapid; meaning that this model simulates an equilibrium reaction at all times. The following graphs are plotted based on the nK value and the four different protein concentrations of BSA and AAG obtained from figure 17 and 18 respectively. Figure 19: Plot of bound drug fraction as a function of BSA concentration (protein-drug binding, relative to Quinidine concentration) ratio using a simplified mathematical model. Affinity is obtained from figure 17. Lines represent drug-binding affinities (product of binding strength and BSA concentration). Shaded area indicates the position within the graph in which physiological BSA to quinidine ratio fall in; ratio 10 to 100. Figure 20: Plot of bound drug fraction as a function of AAG concentration (protein-drug binding, relative to Quinidine concentration) ratio using a simplified mathematical model. Affinity is obtained from figure 18. Lines represent drug-binding affinities (product of binding strength and AAG concentration). Shaded area indicates the position within the graph in which physiological BSA to quinidine ratio fall in; ratio 0. 1 to 1.

## Based on figures 19 and figure 20, it noticeably shows that with increasing protein-to-drug ratio, there is a significant increase in drug bound fraction. It also shows that with increasing protein-to-drug ratio, there is an increase in binding affinity (refer to appendix 3 for the mathematical model data and macros).

At physiological albumin concentrations and under therapeutic concentrations of quinidine (6. 4uM), the protein-to-drug ratio would be within the range of 10 to 100. This would indicate that the protein to drug ratio would have been near the plateau region. As for AAG, the protein-to-drug ratio would fall within the 0. 1 to 1. 0 range; hence quinidine would be found within the steepest region of the curve. This meant that a slight change in AAG concentration or protein-to-drug ratio would result in a significant change in drug binding. Therefore, this would suggest that clinically significant changes in quinidine binding are more likely due to changes in AAG, as patients who have an increased AAG plasma concentration due to an acute myocardial infarction, would have a substantial increase in protein-drug binding; resulting in reduced quinidine interaction at the site of action.[11]It seems that changes in plasma albumin concentration is less likely to cause any extensive changes in drug binding due to several reasons: firstly, HSA has lesser variation in plasma concentration as compared to AAG [11]; secondly, at therapeutic quinidine concentrations the drug falls within the plateau region of the estimated mathematical model, demonstrating that any changes in the protein concentration would not drastically affect drug-protein binding. Using the mean binding parameters for BSA and the mathematical model, it can be predicted that at least a 50% decrease in albumin concentration would be necessary to obtain about 17% increase in quinidine free fraction. This predicted value was very similar to a study conducted by Edwards et al (1983), reporting a 20% increase in quinidine free fraction following a 50% albumin concentration decrease.[11]According to Edwards et al (1983), the mean AAG plasma concentration in patients with trauma or acute myocardial infarction is usually increased to around 1. 97mg/ml, which is ~47uM. He reported that quinidine free fraction was reduced by > 40%.[11] However, based on the mathematical model shown in figure 21, it was predicted that an increase from 18. 3uM (normal AAG plasma concentration) to 47uM of protein would result in a 28% decrease in unbound fraction. This discrepancy in results could be due to the inaccuracy of the AAG nK parameter used in the mathematical model, as the AAG concentrations used in this study was rather narrow (range between 3. 7uM to 19. 2uM), hence the protein-to-drug ratio currently falls within the steepest slope of the graph. Therefore, protein concentration should be increased so that sufficient protein is available for saturation to occur, subsequently allowing a more accurate prediction of protein binding using the protein-drug binding model. Figure 21: Plot of bound drug fraction as a function of AAG concentration (protein-drug binding, relative to Quinidine concentration) ratio using a simplified mathematical model. Comparing two AAG concentrations; one at 47uM (elevated plasma concentration due to myocardial infarction) and the other at 18uM (normal AAG plasma range). Therefore, even though albumin is the most abundant protein in the plasma, it appears that α1-acid glycoprotein is a major protein in the binding of quinidine, agreeing with other studies.[9, 11, 42] Hence, this could possibly mean that removal of albumin might not affect quinidine binding if the concentration of AAG is large enough, but AAG removal could have affected quinidine binding significantly. Since AAG in the plasma is subjected to such great variation, protein binding of quinidine suggests conceivable pharmacodynamics and pharmacokinetics variations in quinidine treated patient.[42] According to Fremstad et al. (1979), he demonstrated a significant positive correlation between the free fraction of quinidine and both volume of distribution and total body clearance,[11, 54] and that increase in bound drug could lead to less drug available for metabolism, prolonged drug clearance and half-life. It seems that in patients with acute myocardial infarction, quinidine might be less effective in reaching the target site, therefore this could be compensated with titrating the dose. However, giving a patient too high a dose could potentially be harmful to the patient as clearance of the drug is extended because protein bound drugs cannot be renally filtrated, hence the drug would accumulate in the body and consequently lead to toxicity. Also, an increase in protein binding can significantly affect quinidine clearance from the body especially when quinidine is cleared mainly via hepatic metabolism, as high protein binding is associated with lowered drug elimination.

## 5. 8 Limitations

This study had created a baseline understanding of quinidine sulphate with the two different proteins, AAG and albumin. Unfortunately, apart from the time constraints, there were several other limitations that hindered a comprehensive understanding of protein binding of quinidine. Firstly, in spite of studies claiming that the degree of protein binding amongst BSA and HSA is often very similar, hence having used BSA in replacement of HSA to understand drug-protein binding;[3] animal proteins do differ from human serum. Such differences include: protein structural variations at the binding sites of the two albumins and quantitative differences with respect to the extent and affinity of drug binding. It has also been reported that significantly lower protein binding has been found in commercially available BSA than that found in HSA. This is probably due to the fact that HSA contains only one tryptophan residue rather than two in BSA.[44] Unfortunately, in this experiment, due to the limited amount of HSA available, BSA was used in replacement. Secondly, in-vitro measurements and predictions of protein binding by just adding physiological concentrations of albumin into a test medium might not be the best representative and model of protein-binding as compared to using pure native serum[44]. This is due to the fact that plasma contains many other proteins, and under physiological conditions, proteins such as lipoproteins might interact with albumin and affect the binding affinity of quinidine to albumin. There might even be competitive binding between the HSA, AAG and other proteins. Thirdly, the mathematical model used in this study was a simplified model that took into account the assumption that the experiment kinetics of binding and dissociation are rapid, though there might be exceptions. Lastly, the results reported in this study were under normal room conditions and not under physiological conditions. Hence apart from the protein concentration, drug concentration and pH of the experiment being kept under physiological conditions; the temperature and the presence of electrolytes were not kept under physiological conditions, which could have potentially affected binding.

## 5. 9 Further improvement studies and work development

As mentioned above, there are many limitations to this project and more can be done to improve the experiment and subsequently, allow better understanding of the interactions of quinidine to the two different plasma proteins. The following recommendations can be proposed, which are based on the current understanding of drug-protein binding and also a build up on this study.(i) While studies in animal serum are valuable, the experiment would be more accurate and relevant if HSA was used instead of BSA, or if the results were supported or compared with findings from HSA.(ii) When drug-protein binding in humans is being investigated, the use of standardised and well-characterised pooled human serum and albumin is preferred.(iii) More variation in protein concentrations should be used, especially for AAG, to allow a complete graph to be plotted from protein to drug concentration ratio 0. 1 to 100. This would provide a better estimate and understanding of the drug-protein binding.(iv) Quantitative measurements and calculation of the bound fraction when variations of both albumin and AAG were added together. This would study the effects of AAG and albumin drug binding in the presence of one another.(v) Further research can be done on varying other factors such as pH, temperature and drug concentration so that a holistic study on the protein binding of quinidine can be done.(vi) Binding of drug to equipment (eg. vials) should be investigated when appropriate to ensure accuracy of the measurement of unbound or bound fraction.

## 6. CONCLUSION

The spectrofluorometry was selected to measure the concentration of free drug because it is a more accurate and sensitive instrument; therefore it is more appropriate as compared to UV-spectrometry for measuring minute amounts of quinidine at physiological concentrations. The time taken for quinidine sulphate to equilibrate in equilibrium dialysis was 4 hours in the absence of protein, and around 5 hours in the presence of protein. Therefore, 5 hours was sufficient to ensure that equilibrium dialysis had occurred. Upon investigating the relationship between protein concentration and the bound-to-free drug ratio, the nK values obtained indicate that AAG has a high binding affinity to quinidine as compared to albumin. It also shows that with increasing protein concentration, there would be an increase in bound drug concentration. With the use of a simplified mathematical model, the protein concentration of both AAG and albumin was varied and the results showed that at physiological concentrations of quinidine, a change in albumin concentration would not have much effect on the bound drug fraction, however, a slight change in AAG plasma concentration would have a significant effect on the bound-to-free drug concentration ratio. Therefore, when there is an increase in AAG plasma concentration due to a myocardial infarction, there would be a significant increase in protein binding of quinidine, which suggests conceivable pharmacodynamics and pharmacokinetic variations; as an increase in bound drug could lead to less free drug available for metabolism, and therefore prolonging drug clearance and half-life. Hence, in patients with acute myocardial infarction, quinidine might be less effective and could be compensated with titrating the dose slightly higher, however at too high a concentration, it could potentially be harmful to the patient.