Uv spectrophotometry calibration curve of quinidine sulfate biology essay

Science, Biology

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.