Plasma / Serum Protein Binding Determinations

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Abstract: The binding of a drug to serum or plasma proteins enables the transport of drugs via the blood to sites of action throughout the body. For expediency we will use serum proteins throughout this discussion with the understanding that one can substitute the term plasma proteins in all experimental instances. Only the fraction of drug unbound from serum proteins is available to diffuse from the vascular system and accumulate in tissues thereby enabling interaction with therapeutic targets and accessibility to xenobiotic clearance pathways. Therefore, the extent of drug binding to serum proteins can have a significant impact on pharmacokinetic (PK) parameters such as clearance rates and volume of distribution. In addition, because only the unbound drug is available to interact with therapeutic targets, the extent of serum binding can have significant effects on the pharmacodynamic properties of a compound as well [1, 2] Determining the fraction of drug bound to serum proteins is a standard parameter evaluated in the process of drug discovery. Although the clinical importance of changes in serum protein binding has been questioned [3-8] the need for serum protein binding studies in the discovery and preclinical development stages is essential for the pharmacokinetic modeling of drugs[1, 3, 9]. The extent of serum protein binding is an important parameter used in many *in vivo* modeling calculations to estimate the volume of distribution, organ clearance, and for scale-up of pharmacokinetic and pharmacodynamic parameters from animal models to humans [3, 10, 11]. The convergence of several trends in the pharmaceutical industry including high speed chemical synthesis technologies, the increasing use of *in silico* ADME modeling together with early *in vivo* evaluations of lead compounds has increased the demand for serum protein binding determinations[12].

Keywords: Equilibrium dialysis, 96-well format, serum protein binding, plasma protein binding, high throughput screening.

METHODS OF DETERMINING THE FRACTION OF DRUG BOUND TO SERUM PROTEINS

Equilibrium dialysis has long been considered the gold standard method for determining the fraction of drug bound to serum proteins. The equilibrium dialysis approach has a low potential for experimental artifacts and allows one to determine the extent of binding to the entire spectrum of serum proteins. The 20 cell equilibrium dialyzer manufactured by Spectrum (www.spectrapor.com) and Dianorm (Germany), are industry standards to which novel dialysis approaches have been compared. However, these gold standard systems have several drawbacks which limit their application in the current high throughput pharmaceutical environment. These original methods required relatively large sample volumes (1ml) with the concomitant need for more compound and serum. In addition, these original systems required extensive equipment setup times resulting in low compound throughput capabilities. For example, when using the Spectum 20 cell system, samples are inserted and withdrawn from the dialysis cells using syringes, making it less adaptable to laboratory automation. Although equilibrium can be reached in these systems within six hours, most investigators dialyzed overnight because the time required to setup the dialysis cells, load, and then retrieve the samples generally requires an excess of 8 hours. These original systems were also subject to volume shifts from the buffer to the serum side of the dialysis cell, effectively diluting the serum proteins and requiring mathematical corrections to estimate the fraction of compound bound in undiluted serum [13].

Several alternative methodologies have been developed to address the limitations of these original equilibrium dialysis devices. The ultrafiltration method has been a popular alternative and filtration devices are commercially available from several vendors (www.millipore.com, www.amicon.com and www.sartorius.com) [14]. This approach eliminates the extensive setup time required for the original equilibrium dialysis equipment and reduces the time needed to generate biological samples for quantitative drug analysis. Minimizing the time to generate biological samples is an advantage especially for compounds which are metabolically unstable in serum. However, the quality of the data from this technique is notoriously dependent on the extent of nonspecific binding of the test compounds to the polystyrene apparatus or the ultrafiltration membrane surface [1]. The nonspecific binding effects are particularly challenging for drugs highly bound to serum proteins. This drawback has been partially overcome by recent modifications to ultrafiltration methodology, (i.e. mixing of control plasma or serum retentate with the filtrate), thus reducing the nonspecific binding effect [15, 16] or pre-treatment of the filter membrane with Tween 80 or benzalkonium [17]. Another constraint associated with the ultrafiltration approach is the prerequisite to only collect the minimum amount of ultrafiltrate required for analysis because concentration of serum proteins during centrifugation may increase the apparent fraction of drug binding to serum proteins [18].

A second alternative method is to determine the binding of compounds to human serum albumin (HSA) and/or alpha-1 acidic glycoprotein (AGP) serum proteins immobilized on HPLC columns [19]. In general, it has been observed that acidic drugs tend to bind preferentially to HSA and basic and neutral drugs to AGP [20]. Immobilized HSA columns have also been utilized to determine protein binding kinetics which have been shown to impact pharmacokinetic profiles [9, 21]. A disadvantage of this approach is that one can only measure binding to the specific proteins immobilized on the column while human serum contains >60 different proteins with a wide range of concentrations and binding affinities/capacities. This approach is justified in part by the fact that HSA and AGP are the most abundant proteins in plasma and account for the majority of compound bound to serum proteins [5, 15, 22]. HSA and AGP columns are currently only available for a limited number of species. The advantages and disadvantages of approaches using HSA and AGP HPLC columns have recently been reviewed in detail by Wan and Bergstrom[15]. The remainder of this review will focus on developments and modifications to the equilibrium dialysis method which have increased throughput while minimizing assay resource requirements.

FUNDAMENTALS OF EQUILIBRIUM DIALYSIS

In an equilibrium dialysis experiment, the dialysis cell consists of two chambers ("D" and "R" in Fig. **1**) separated by a dialysis membrane. The dialysis membrane is semi-permeable in that it

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1389-2002/08 $55.00+.00

contains pores that are large enough to allow small molecules like drugs to move freely back and forth but too small to permit the movement of larger molecules like serum proteins to pass. Serum spiked with drug is added to one side of the dialysis membrane ("D") and buffer or serum ultrafiltrate is added to the other ("R"). At equilibrium, some fraction of the drug remains bound to the proteins on the serum or donating side of the dialysis cell while the concentration of drug unbound from serum proteins or free drug is equivalent on both sides of the dialysis membrane. The fraction unbound (fu) is equal to the concentration of free drug divided by the total concentration of free plus bound drug. The concentration of drug on the serum side of the dialysis cell ("D") is equivalent to the sum of the bound plus unbound drug while the concentration of drug on the dialysate or receiving side of the dialysis cell ("R") is equivalent to the unbound drug concentration. The fraction unbound (fu) is therefore equal to the concentration of drug on the dialysate side (the free drug concentration) divided by the concentration of drug on the serum side (concentration of bound plus free drug) or fu=R/D.

Although there is no industry standardized protocol for determining serum protein binding via equilibrium dialysis, there are many factors to consider that can impact serum protein binding [1, 23, 24]. Similar results have been obtained using fresh or frozen stocks of plasma or serum [25]. However if one chooses to use frozen stocks, it is generally easier to work with serum as plasma tends to form fibrinogen precipitates following a freeze thaw cycle which subsequently clog pipette tips. Many drugs exhibit pH dependent binding to serum proteins that can have significant effects on fu in either *in vitro* or *in vivo* environments [26]. Therefore, serum pH should be adjusted to 7.4 prior to use via the addition of phosphoric acid or by purging with CO2. Incubating serum in an air incubator or water bath for long periods of time (> 12 hours) can result in a 1-1.5 unit shift in pH [2]. This is due to the evaporation of CO2 from the serum and can be compensated for by either incubating in a CO2 incubator at 37°C (personal communications) or by using alternative buffering systems with higher buffering capacity [2]. Restricting exposure to air either by using a closed system or covering the top of the dialysis apparatus with adhesive film can also significantly reduce changes in pH. The impact of pH-induced changes on drug binding to serum proteins and the potential clinical relevance has recently been reviewed [26, 27].

It is ideal to evaluate serum protein binding around projected clinical exposure ranges. In situations where this value is not known, drugs can be evaluated in the 5-10μM range. The analytical sensitivity of drug detection in the free fraction, especially for highly bound compounds will establish the lower limit of testing for many drugs. There is also considerable sample-to-sample variation of total protein concentrations in human plasma as well as at least 18 different variants of HSA which have been identified. Therefore, use of pooled human serum samples is essential to minimize experimental variability. In addition, there are significant differences between species regarding total serum protein concentrations as well as the abundance of individual proteins and protein variants. This requires that the appropriate species be chosen for each experiment.

Dialysis membranes, made of regenerated cellulose, are available in a range of nominal pore sizes (www.spectrapor.com, www.visking.com, www.HTDialysis.com). The pore size of the membrane as well as the membrane thickness impacts the time required to reach equilibrium. In general, the time required to reach equilibrium is governed by the ratio of the exposed surface area of membrane to the sample volume and can be reduced by using thinner membranes or membranes with larger pore sizes. Also, high molecular weight, less compact or highly hydrophobic compounds may require additional time to pass through the hydrophilic dialysis pores. Most dialysis membranes are packaged dry and it is advisable to always rinse cellulose membranes prior to use to remove any adherent impurities. The specified membrane pore size is generated when the membrane is hydrated. We have traditionally hydrated membranes overnight in 50% PBS: 50% EtOH and rinsed membranes prior to use in phosphate buffered saline (PBS). Hydrating dialysis membranes in 50% EtOH helps to eliminate the potential for microbial digestion of the cellulose membrane and facilitates removal of glycerol which is added to cellulose membranes during manufacturing to promote efficient membrane hydration and limit desiccation during storage. One advantage of hydrating overnight is that the membranes are available for immediate use in the morning, permitting 6 hours for dialysis and 2 hours for sample loading and collection enabling one to complete experiments within an 8 hour workday. To ensure membrane integrity, hydrated membranes should never be allowed to dehydrate before use.

A typical equilibrium dialysis experiment is initiated by first setting up the dialysis wells partitioned by the dialysis membrane of choice. An isotonic buffer or serum ultrafiltrate is added to one side of the dialysis cell and plasma or serum spiked with test compound (5-10μM) to the other side of the cell. The samples are incubated at 37°C in a pH controlled environment until equilibrium is attained. Depending on the chemical properties of the test compound, physical characteristics of the dialysis apparatus, and the membrane used, equilibrium is frequently established within 4-6 hours. The time to reach equilibrium can be modestly reduced by rocking or rotating the apparatus. The chemical properties of a test compound can significantly impact the time required for the system to reach equilibrium. For example, highly lipophilic or hydrophobic compounds may require additional time to pass through the hydrophilic pores in the dialysis membranes. Equilibrium time dependence should be addressed by conducting a control experiment to determine the length of time required to reach equilibrium for each test compound or compound series prior to the serum binding experiment. This can be accomplished by spiking test compound into isotonic buffer or serum ultrafiltrate and dialyzing against the un-spiked equivalent.

In the original equilibrium dialysis systems, the presence of serum proteins on the serum but not the dialysate side of the dialysis membrane causes a difference in osmotic pressure which resulted in a net movement of buffer from the dialysate side to the serum side of the dialysis cell. This is referred to as a "volume shift". In our laboratory, following 8 hours of dialysis, serum in the sample side of the Spectrum 20 cell dialyzer was diluted to 87% of its original concentration [18]. This volume shift reduces the concentration of serum proteins, thereby reducing the on rate of the on/off binding [13] equilibrium between the drug and serum proteins resulting in an increase in the observed fraction unbound. Similar dilutions of plasma have been shown to reduce the fractional binding of compounds to plasma components [28]. In the past, clinical grade dextran (molecular weight, 81,600) has been added to the dialysate side to equalize the osmotic pressure and help minimize the volume shift [28, 29]. The need to demonstrate that the test compound does not bind to dextran makes this approach less attractive for higher throughput applications.

Mathematical formulas have been developed to help compensate for the decrease in fractional binding caused by the volume shift [13, 30]. The fraction of drug bound to serum proteins is reduced by a proportion related to the extent of the volume shift. Derivation of the Boudinot formula (Fig. **2**) shows that for all compounds, the concentration of drug bound is corrected by the factor "Vpe/Vpi" where "Vpe" represents the volume of sample at equilibrium and "Vpi" represents the initial volume of sample before the start of dialysis. The "Vpe/Vpi" correction factor is intended to compensate for the decrease in fraction bound caused by the dilution of plasma during dialysis [13]. This mathematical formula assumes that the fractional plasma binding of all compounds is equally affected by the dilution of plasma components. However, theoretical simulations have shown that osmotic fluid shifts and associated serum dilution produce the largest changes in fractional

binding for compounds that are bound by low capacity proteins with low affinity constants [13]. A study conducted to determine the effect of serum dilution on the fractional binding of 10 compounds in serum at 100, 85 and 70% of the original serum concentration demonstrated that the fractional binding of some compounds was more sensitive to serum dilutions than others [18].

More recently, several 96-well equilibrium dialysis apparatuses have been developed and are now available from commercial sources (www.HTDialysis.com, www.piercenet.com www.harvardapparatus.com) [18, 31]. A prototype of the re-usable 96-well dialysis block, currently marketed by HTDialysis LLC, was initially developed in our laboratory to address our needs as a centralized provider of serum protein binding evaluations. The key requirement during development of this 96-well dialysis apparatus was that the apparatus needed to be compatible with all standard 96-well formats, laboratory supplies and robotic instruments. It was constructed of virgin Teflon to minimize the potential for nonspecific binding of test compounds to the apparatus. The design has a 30 – 150ul working volume that maintains a constant membrane surface area to volume ratio. Low volume (100µl range) was essential to minimize compound usage as well as reducing serum requirements for studies involving smaller animal species. To facilitate automation, the apparatus was designed for robotic access to both the sample and dialysate sides of the dialysis apparatus from the top of the dialysis block. The dialysis membrane was aligned vertically in the well to maximize the surface to volume ratio, simultaneously eliminating problems associated with trapped air bubbles while permitting the investigator to add or remove samples from each well independently or all wells at once. The system needed to be easily scalable to enable analysis of a large number of samples, time points, or replicates in the same experiment. This vertical design is also available in the Pierce Biotechnology "Rapid Equilibrium Dialysis" (RED) Device.

For all three common methods of determining serum protein binding values (equilibrium dialysis, ultrafiltration and binding to serum protein columns) developing methods for detecting each individual analyte and their concentration is the most resource intensive aspect of serum binding determinations. The sensitivity of the detection system to the analyte, especially for highly bound compounds, is frequently the limiting factor which determines the concentration compounds need to be evaluated at. This need for high sensitivity has resulted in many laboratories utilizing LC/MS/MS for quantitation. Drawbacks to the LC/MS/MS approach include the need for developing MS detection methods for each analyte and the influence the matrix environment has on the ionization potential for each analyte [32]. The potential matrix effect has historically required investigators to compare the LC/MS/MS analysis of a standard curve prepared using test compounds spiked into serum precipitated with 3 volumes of acetonitrile (ACN) to a second standard curve using test compound spiked into the complementary isotonic buffer. This approach doubles the number of samples requiring analysis.

MODIFICATIONS TO POPULAR SERUM BINDING METHODOLOGIES

Most common serum binding protocols use some version of an isotonic buffer (i.e. PBS) on the dialysate side of the dialysis cell to both maintain normal ionic strength and limit pH fluctuations which could alter the serum protein binding characteristics of the test compound. One adjustment to the standard protocol adopted by our laboratory was to replace the isotonic buffer with serum ultrafiltrate. There were several variables associated with using isotonic buffer that we hoped to control by introducing the serum ultrafiltrate as the dialysate medium. Primarily, there are many molecular components in serum which are small enough to pass through the 12-14K molecular weight cut-off membrane pores traditionally used for equilibrium dialysis. At equilibrium, the dialysate consists of the initial buffer modified with the dialyzable serum components and has different properties than the starting isotonic buffer. This modified dialysate buffer is a different matrix that may impact the ionization potential of the analyte during LC/MS/MS analysis necessitating creating a standard curve in modified buffer as well. We also observed that some compounds were more soluble in the post dialysis modified PBS than in PBS used to make the standard curve samples. Serum ultrafiltrate is made by passing serum through a 10K MWCO Microcon filtration device (Millipore Cat # YM-10). Serum components which pass through the 10K MWCO filter mimic those that pass through the 12-14K MWCO pores during dialysis. Switching from PBS to serum ultrafiltrate, permitted us to reduce the potential for volume shifts, improve compound solubilization in standard curve samples and create a common matrix for the standard curve and dialysate samples improving the accuracy and reducing the number of samples for LC/MS/MS analysis.

Another change to enhance analysis was an adoption of compound sampling and dilution prior to LC/MS/MS analysis. The primary objective was to create a common matrix for both serum and dialysate samples enabling all results to be correlated to a single standard curve thereby reducing the total number of samples for analytical evaluation (Scott Obach - personal communication), [33, 34]. Serum and dialysate samples were uniquely diluted to reduce dependence on separate standard curves to calculate analyte concentrations while enabling one to use direct MS signal intensities for routine analysis. At equilibrium, one removes 10µl of sample from the serum side and adds 90µl of fresh serum ultrafiltrate (10-fold dilution) followed by precipitation with three volumes (300µl) of ACN. Conversely, one removes 90µl of sample from the dialysate side and adds 10µl of fresh serum (1.1-fold dilution) followed by precipitation with three volumes (300µl) of ACN. Samples used to generate the standard curve are generated by diluting the test compounds in a 90/10 mixture of fresh serum ultrafiltrate and serum, respectively, followed by precipitation with 3 volumes of ACN. Following this protocol, results in all samples have a common matrix with a constant serum protein concentration. In addition, if the fraction unbound for the test compound is 10%, then the MS signal generated by diluted serum and dialysate samples will be the same. This effectively improves the accuracy of the assay by reducing errors associated deviations in the standard curves for compounds in the higher protein binding ranges where such errors are of higher significance to drug development efforts.

The availability of an equilibrium dialysis apparatus compatible with standard 96-well formats has significantly reduced the resource required to generate biological samples for analytical analysis. For projects requiring larger numbers of test compounds to be evaluated, all manipulations can be accomplished using either 96-well pipetting systems (e.g. Personal Pipettor - www.apricot designs.com., Quadra 96 – www.tomtec.com) or adapted to various robotic pipetting platforms (e.g. Tecan Group Genesis www.tecan.com, Packard MultiPROBE - www.perkinelmer.com, Beckman Biomek- www.beckmancoulter.com, or Hamilton –STAR - www.hamiltonrobotics.com). The rate limiting or most resource intensive step required for protein binding determinations using equilibrium dialysis continues to be quantitation of analyte in the biological samples. Even using the simplified approach described above, one still needs to analyze about 14 samples per test compound to include duplicate samples, controls to show equilibrium was achieved, and a standard curve to demonstrate that the LC/MS/MS signal is dose dependent. Numerous advancements in analytical technologies including automated methods development programs and high speed injection/column switching systems associated with robotic decks have significantly reduced the resource required to evaluate samples in 96-well formats [35-38]. In addition, Wan *et al.* have developed a sample pooling method to further reduce the number of bioanalysis samples. These modifications combined with rapid generic LC/MS/MS methods development

have significantly reduced the resource required to determine the concentration of analyte in biological samples [2, 15].

To further address the higher throughput and lower cost requirements for serum protein binding determinations conducted in the early drug discovery setting, we have developed an alternative formula for calculating fu values. This new formula is compatible with existing methodologies as all the same biological samples are used. However, analytical quantitation of only a subset of the samples is needed to reach the level of accuracy required for the early stages of drug discovery. In the standard formula fu= R/D, "D" is the concentration of bound plus free drug on the donating or serum side of the dialysis well at equilibrium and "R" is the concentration of free drug on the receiving or dialysate side of the dialysis well (Fig. **1**). As described above, one should always include an experimental control to demonstrate that the samples have been dialyzed long enough for equilibrium to be achieved. This is accomplished by spiking test compound into isotonic buffer or serum ultrafiltrate and dialyzing against the same. These equilibrium control samples are represented as samples "A" and "B" in Fig. (**1**). If the system has been incubated for a sufficient length of time, the concentration of test compound in samples "A" and "B" should be equivalent or A/B=1. The A and B samples can be prepared for analysis by diluting 50µl of sample (A or B) with 40µl of clean serum ultrafiltrate and 10µl clean serum prior to precipitation with 3 volumes of ACN to generate samples with a common matrix and similar compound concentrations as the diluted "R" and "D" samples described above (Fig. **1**).

The conceptual and mathematical derivation of the novel formula is outlined in Fig. (**3**). In short, the formula utilizes test compound concentration data from only two of the four dialysis samples. First is the unbound drug concentration "R" from the dialysate side of the compound spiked into serum where the "R" sample is the same sample used in the standard formula fu=R/D (Fig. **1**). Second is the drug concentration in sample "B" from the equilibrium control experiment where compound is spiked into serum ultrafiltrate and dialyzed against unspiked serum ultrafiltrate (Fig. **1**). The algebraic rearrangement (shown in Fig. **3**), demonstrates that the concentration of test compound in sample "D" should be equivalent to "2B-R". One can then replace the value "D" in the equation "fu=R/D" with "2B-R" resulting in the Banker formula "fu=R / (2B-R)". The new formula assumes that if drug is lost in the system due to nonspecific binding or because the test compound has not yet completely reached equilibrium (i.e. (A>B) in the equilibrium control experiment), then a similar event will occur in the complementary dialysis well where the test compound was spiked into serum. The concentration of test compound in sample "B" represents the amount of compound one could expect on the dialysate side if there was 0% binding to serum proteins and also accounts for nonspecific binding and events where equilibrium has not been fully achieved. When using the fu=R/(2B-R) formula, one now only has to determine the concentration of test compound in two samples per replicate to generate fraction unbound values which also factor in any loss of test compound due to nonspecific binding to the dialysis apparatus or experiments which were not dialyzed for a sufficient length of time to completely reach equilibrium.

The biological samples required to utilize this novel formula are a subset of samples used in the standard formula. This allows an investigator to continue running the standard assay format, collecting and analyzing each of the "R", "D", "A", and "B" samples (Fig. **1**) using the formula fu=R/D to calculate fraction unbound and the ratio of A/B to confirm that the system has been incubated long enough to reach equilibrium. One can then use the available values to re-calculate fraction unbound using the formula fu=R/(2B-R) until a sufficient level of confidence has been established. In the case described above, where fraction unbound is calculated using both formulas (fu=R/D and fu=R/(2B-R), experiments where the two values are significantly different can serve to flag results for further review. In most cases, where significantly different results were obtained using the two formulas, we also observed challenges with compound solubility.

The real value of the using the fu=R/(2B-R) formula occurs when one has established sufficient confidence to analyze only the "R" and "B" samples. Since "R" and "B" samples are serum ultrafiltrates (no serum proteins), these samples can be directly injected onto LC/MS/MS systems, eliminating the need for ACN precipitation. Chromatography and column sample switching technology can also be used to shunt the void volume (containing salts) to waste prior to injecting the sample into the ionization chamber. This protocol modification significantly reduces sample handling requirements and allows the entire operation to be automated whereby 96-well tube racks containing compounds, serum and serum ultrafiltrate along with the 96-well equilibrium dialysis apparatus can be loaded onto robotic decks. Samples could be loaded into and removed from the dialysis block directly on the robotic deck and since no ACN precipitation step is required, the sample can be injected directly into LC/MS/MS for quantitation.

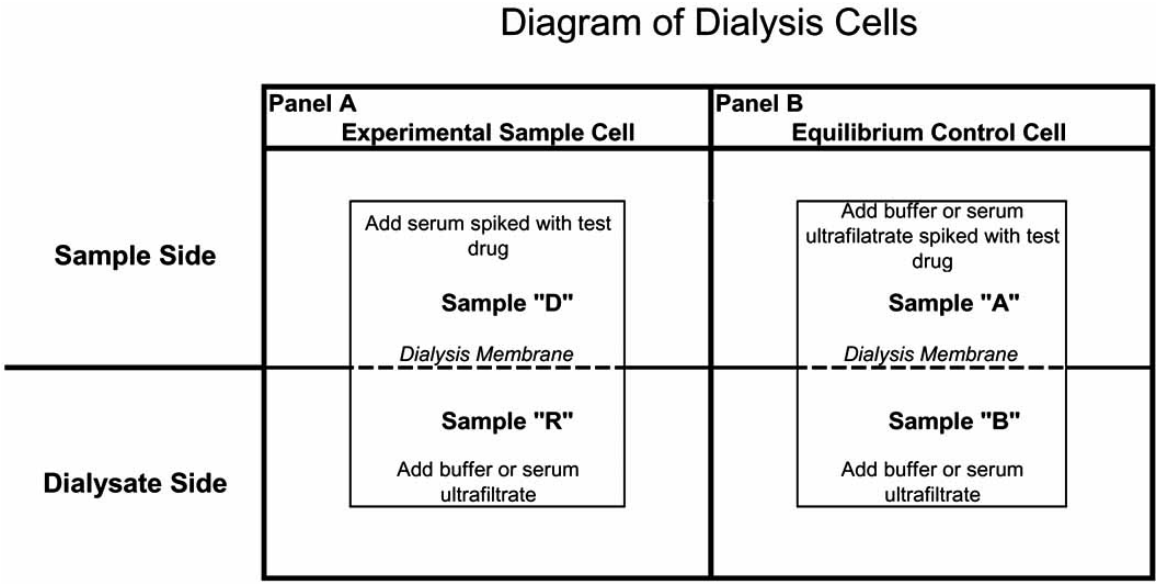

Fig. (1).

Derivation of Boudinot Formula

A. Fraction Bound $= \dfrac{\text{[drug bound] (corrected for volume shift)}}{\text{[Total drug]}}$

B. Fraction Bound $= \dfrac{\text{Drug Bound} * \dfrac{\text{volume of plasma (at equilibrium)}}{\text{volume of plasma (initial)}}}{\text{Drug Bound} * \dfrac{\text{volume of plasma (at equilibrium)}}{\text{volume of plasma (initial)}} + \text{[free drug]}}$

C. [drug bound] = [total drug] - [free drug]

= [drug on plasma side at equilibrium]- [drug on buffer side]

D. Fraction Bound $= \dfrac{(DTe\text{-}DF) * \dfrac{Vpe}{Vpi}}{(DTe\text{-}DF) * \dfrac{Vpe}{Vpi} + DF}$

Fig. (2). Derivation of the Boudinot formula for calculating fraction bound.

Derivation of Novel Formula for Calculating Fraction Unbound

Original equation (assuming no volume shift)	Fu= [R]/[D]
When equivalent amount of analyte is added to each the experimental and equilibrium control cells	[R] + [D] = [A] + [B]
When the control cell reaches equilibrium	[A] + [B] = 2[B]
Substitute"2[B]" for "[A]+[B]"	[R] + [D] = 2[B]
Solve equation for "[D]"	[D] = 2[B] - R
Substitute "2[B]-R" for "D" in original equation	Fu= [R] / (2[B]-[R])

Fig. (3).

CONCLUSION

Advances in high speed chemical synthesis technologies combined with the desire to predict human ADME parameters earlier in the drug discovery process is placing an increased demand on the need for serum protein binding, microsome protein binding [10, 11, 39, 40] and tissue binding data to assist in early PK/PD modeling. Equilibrium dialysis is well established as the preferred approach to generate this information during the final stages of drug development when precise information is required for a limited number of test compounds. At this later stage of drug development, one can utilize well established equilibrium dialysis methodologies, complete with therapeutically relevant drug exposures, analysis of relevant species, replicates, internal controls and standard curves to calculate the drug fraction unbound from serum proteins. However, the high resource requirements and the limited throughput capacities associated with the historical equilibrium dialysis methodologies have encouraged efforts to develop alternative methodologies to determine serum/plasma protein binding values for use in the early drug discovery setting. We have outlined an approach whereby equilibrium dialysis can be implemented at the earliest stages of drug discovery efforts where the need for precise fraction unbound values is limited by the level of precision in other ADME parameters utilized for modeling. During this early drug discovery stage, it is more important to be able to quickly rank order compounds within and across chemical series in order to help prioritize a subset for further advancement. It is also important to have the ability to rapidly evaluate drug binding to serum of multiple relevant species to help develop and understand PK/PD modeling efforts. The development of commercially available 96-well equilibrium devices which are compatible with standard 96-well pipetting instrumentation and robotic liquid handling systems, makes generating the biological samples required for analysis using equilibrium dialysis facile and cost effective. Automated LC/MS/MS methods development for individual analytes along with high speed analytical methodologies allows rapid quantitation of analytes in biological samples [36-38]. Using the techniques described above, one can adjust the level of resource used to generate serum protein binding data points by reducing the most resource intensive step, quantitation of individual compounds. In the earliest stages of drug discovery, direct injection of just two samples generates a reasonable and reliable estimate of serum protein binding. At the final stages of drug discovery, one can include replicate samples, controls to demonstrate equilibrium was reached and standard curves to generate fraction unbound values using regulatory agency approved methodologies.

Adopting this approach does not require an unsubstantiated leap of faith but rather an experimental progression. The less resource intensive approach calculates the fraction unbound values using a subset of the biological samples evaluated for the most stringent analysis. Therefore, one can begin by using the data routinely generated under the most stringent applications to recalculate fu values using the less resource intensive formulas. Experimental data will permit rational reduction of the number of samples requiring analytical quantitation. Generation of all the biological samples needed for detailed analysis is facile and by selective bioanalysis, the level of accuracy needed at each stage of drug development can be modulated. A strategy of statistically spot checking a subset of samples will inspire confidence in the approach and help reassure

colleagues of the data quality. The gold standard approach, equilibrium dialysis, can be used at all stages of drug discovery to generate serum/plasma protein binding values by adjusting the number of biological samples requiring analytical quantitation (via number of replicates, internal controls, standard curves, compound pooling) so that the resource required to generate the data point does not exceed the value of the information.

ACKNOWLEDGEMENTS

We would like to thank Adam Brockman for his assistance in generating Fig. (**3**), which demonstrates the algebraic rearrangement required to derive the novel formula for determining protein binding values. We have especially enjoyed the continuous support and encouragement provided by John Williams and Dennis Pereira throughout this project.

ABBREVIATIONS

CAN = Acetonitrile

AGP = Alpha-1 acidic glycoprotein

Fu = Fraction unbound

HAS = Human serum albumin

MWCO = Molecular weight cut off

PD = Pharmacodynamic

PK = Pharmacokinetic

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Received: April 08, 2008 **Revised: July 27, 2008** **Accepted: July 28, 2008**