Plasma Protein Binding in Drug Discovery and Development

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Abstract: This review describes methods for quantifying the binding of small molecule drug candidates to plasma proteins and the application of these methods in drug discovery and development. Particular attention is devoted to methods amenable to medium-to-high throughput analysis and those well suited for measurement of compounds that are highly protein bound. The methods reviewed herein include the conventional techniques of equilibrium dialysis, ultrafiltration and ultracentrifugation, as well as some more novel approaches utilizing micropartitioning and biosensor-based analysis. Additional concepts that are discussed include plasma protein structure, enantioselective protein binding, drug displacement, the effect of patient demographics and disease states on free (unbound) drug levels, and the influence of protein binding on drug candidate pharmacokinetics and pharmacodynamics. Practical considerations pertaining to the evaluation of highly protein bound drug candidates are also highlighted.

Keywords: Plasma protein binding, human serum albumin, pharmacokinetics, equilibrium dialysis, ultrafiltration, ultracentrifugation, optical biosensors and micropartitioning.

PHARMACOKINETIC AND PHARMACODYNAMIC CONSIDERATIONS

The pharmacological properties of drugs are often directly related to the free drug concentration in plasma. This notion rests on the concept that only unbound drug is available to passively partition into the site of action to interact with the molecular target. Correspondingly, bound drug cannot passively diffuse to the active site and is therefore unable to interact with the target. This concept is reflected by the pair of equilibrium equations given below,

$$\begin{array}{ll} D + PP \leftrightarrow DPP \text{ (Plasma compartment)} & Ka = [DPP]/[D]\,[PP] \\ \updownarrow & (1) \\ D + R \leftrightarrow DR \text{ (Site of action)} & Ka' = [DR]/[D]\,[R] \end{array}$$

where D is free drug, PP are plasma proteins, DPP is drug bound to plasma proteins, R is the molecular target at the site of action, and DR is target-bound drug. Although the equilibrium constant for DR formation (Ka') can be orders of magnitude larger than that for plasma protein binding (Ka), for passively diffused drugs the concentration of free drug at the site of action will still be limited by free drug concentration in plasma. However, if migration of free drug to the molecular target is mediated by processes other than passive diffusion, such as the presence of a pH gradient across a membrane that may affect drug solubility or the occurrence of active cellular efflux or influx involving transporters, the formation of DR, and hence the biological response, may not be a direct function of free drug concentration in plasma. In these instances, the equilibrium equations will still hold, but the rate of DR formation may no longer be limited by free drug diffusion to the site of action but rather by the rate of active transport and/or efflux. A notable example of this is the efflux of drugs at the blood brain barrier prohibiting their migration into the central nervous system. Similarly, the biological action of drugs that are actively transported into the brain can be described by more complex equilibrium models that may depend on the rate of influx, the off-rate of drug bound to plasma proteins, as well as rates of elimination or metabolism of the drug in the brain compartment [1]. Such detailed pharmacokinetic/ pharmacodynamic (PK/PD) models, which predict and explain biological responses as a function drug concentration (whether free or total concentration), are becoming increasingly important in drug R&D [2], helping researchers choose lead candidates, set optimal dose regimens, and interpret clinical results.

Clearance of drugs from the body can be influenced by the extent of protein binding, but the trend will depend on the route of elimination. For example, the extent of plasma protein binding may enhance elimination by supplying the drug to the liver *via* blood flow while limiting distribution to systemic tissues. On the other hand, renal clearance through glomerular filtration can be slowed by extensive plasma protein binding by essentially excluding drugs from filtration [3]. Elimination of drugs by the liver can be described in terms of multiple equilbria and rate constants to define the relationship between plasma and intracellular free drug concentrations. In one such model [4], plasma protein binding, passive diffusion into the hepatocyte, active transport into and out of the hepatocyte, binding to cellular proteins, metabolism, and active efflux of drug into bile are simultaneously taken into account with both the passive diffusion and active influx of the drug into the hepatocyte being dependent on free drug concentration in plasma. The model is able to correct for the underestimation of hepatic clearance that often occurs when drugs are highly protein bound and/or transported into hepatocytes.

The volume of distribution is also a function of free fraction, as described by the following expression:

$$V = Vp + (fu/fu_t)\,Vt \quad (2)$$

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In this equation, fu represents the unbound fraction of drug in plasma, fu_t represents the unbound fraction of drug in tissue, Vt is the tissue volume, Vp is the plasma volume and V is the total apparent volume of distribution. When V is large (>30L), Vp will be small relative to the total distribution volume. In this case, V will approach (fu/ fu_t)Vt and will be affected by changes in free drug fraction. If, on the other hand, V is small and approaches Vp, then V is essentially independent of fu. As seen in equation 2 above, V will be dependent not only on free fraction in plasma but also on free fraction in tissue. Since free drug fraction in tissue is not readily determined and may vary between species, human V cannot always be predicted using animal data [5].

Drug Candidate Free Fraction Evaluation *Ex Vivo*

Plasma protein binding (PPB) of drug candidates can be measured *in vitro* using plasma from multiple species, including human. Comparison of free fraction across species and over a range of drug concentrations is helpful in interpreting preclinical PK and PD results and in predicting the properties of a drug candidate in humans. The measurement of PPB is often performed in conjunction with red blood cell (RBC) partitioning in that both partitioning of free drug into RBC and PPB will affect the free drug concentration available for target binding and, potentially, the pharmacological effect. Analogous to PPB measurements, the RBC screens are also performed in multiple species over a range of drug concentrations. Plasma protein binding and preferential partitioning of drugs into RBC are important factors in setting the initial dose of the drug in Phase I clinical trials.

PLASMA PROTEINS INVOLVED IN DRUG BINDING

Human Serum Albumin

Plasma contains various proteins that function as carriers of endogenous and exogenous molecules throughout the circulatory system. The plasma proteins most often associated with the binding of small molecular weight (MW) drug molecules in plasma are albumin and α-1-acid glycoprotein (AAG) and, to a lesser degree, globulins and lipoproteins [6, 7]. The relative significance of these proteins for drug binding is determined by both the presence of specific high-affinity binding sites contained on the proteins and the protein abundance.

At concentrations of 500 to700 μM [6], human serum albumin (HSA) is the most abundant protein in human plasma and helps maintain osmotic pressure and pH in the blood stream. Human serum albumin acts as a carrier for drugs, peptides, fatty acids, bilirubin and other endogenous compounds [8]. Small MW molecules that form non-covalent complexes with HSA can be shielded from certain elimination pathways such as glomular filtration of the kidneys and enzymatic reactions in the liver and blood-stream. Analogous to protein receptor/ligand interactions, the reversible high-affinity binding of a small MW drug candidate to plasma proteins is dictated by specific molecular interactions between the drug candidate and amino acid residues that create the protein binding site. Although a number of exceptions exist, anionic compounds tend to bind specifically to HSA whereas those that exhibit cationic, or basic, properties predominantly bind to AAG [9]. In general, the degree of protein binding increases with the hydrophobic character of the compound [10].

Human serum albumin is a nonglycosylated monomeric protein containing 585 amino acids, 17 disulfides and one free sulfhydral cysteine at Cys_{34}. The structure is characterized by 67% α-helix, 23% extended chain and 10% β-turn [8]. It has a high degree of ionic residues resulting in high water solubility and is highly flexible, resulting in the ability to specifically bind a large array of molecules. The atomic structure of HSA as characterized using x-ray crystallography by He and Carter [11], is described as a heart-shaped protein with three homologous domains (labeled I, II and III) that can each be further divided into two subdomains (labeled A and B) having similar structure. There are at least two high affinity drug binding sites on albumin as well as many low affinity sites. The two high affinity sites most often associated with drug binding have been characterized and named based on drugs that they bind. They are most commonly known as Site I, or the warfarin binding site, and Site II, or the benzodiazepam binding site [12]. A lesser studied high affinity binding site on albumin has been termed site III and has been shown to specifically bind digitoxin [13].

The interactions of drugs with albumin has been extensively described based on albumin structure by Ghuman *et al.* [14]. In this paper, drug-binding sites were characterized using x-ray crystallographic analysis of albumin bound to 12 different drugs or toxins with and without fatty acids present. Site I, located on subdomain IIA, was characterized as having mainly hydrophobic residues throughout the binding site with basic or polar residues at the entrance and toward the bottom of the pocket [14, 15]. Consistent with this observation, compounds having two acidic moieties spaced 5 to 6 bonds apart that can interact with these two basic regions have shown high affinity binding (Kd = 100 nM). Fatty acid binding to fatty acid site FA2, was shown to dramatically change the site I volume and orientation of the binding residues causing disruption of drug binding. Contrary to fatty acid binding, which imparts global conformational changes, the binding of drugs to either site I or II induce only local conformational changes near the binding site. Structural characterization of binding site II, contained in subdomain IIIA, showed a binding pocket that is topologically similar to site I, but smaller in size and having only one polar area located at the pocket entrance. This observation is consistent with the structure of site II binding compounds that are largely hydrophobic with a peripherally located electronegative structural group.

The observations made in this and other crystallographic studies of HSA alone [15], or complexed with fatty acids [14, 16, 17] or with various drugs [14, 18, 19], have advanced the understanding of drug-albumin interactions on a molecular level. This knowledge contributes to a better picture of the specific nature of drug-albumin interactions and supplies the medicinal chemist with a quantitative understanding of the drug-albumin binding process. Such information may be used to help predict and manipulate protein binding during drug design.

Alpha-1-Acid Glycoprotein

The next most important plasma protein responsible for drug binding is α-1-acid glycoprotein (AAG), also known as orosomucoid. AAG is an acute-phase protein having levels that are modulated in certain pathological conditions such as cancer, cardiovascular disease, inflammatory disease, kidney disease, liver disease and infections. AAG expression has been shown to increase in response to inflammatory and immunological stress and is modulated by glucorticoids and cytokines, including, IL-1, IL-6 and possibly IL-8 [9, 20, 21]. The protein itself has an approximate MW of 41-45 kDa and is heavily glycosylated with about 45% of its mass comprised of carbohydrate [20]. The extensive glycosylation results from five N-linked glycans that are extensively sialylated giving the protein a net negative charge at neutral pH (pI = 2.7 -3.2) and a high degree of water solubility [20]. The three dimensional structure of AAG has been studied by Raman and infrared spectroscopy at pH 7.4 and contains 15% α-helices, 41% β-sheet 12% β-turn, 8% band and the remaining unordered structure [22].

Although the physiological function of AAG is not well understood, it has been shown to act as an immunosuppressive and immunomodulating agent, as well as an inhibitor of platelet aggregation, neutrophil activation, lymphocyte proliferation, and IL-2 secretion [21]. Under normal physiological conditions the concentration of AAG in serum is about 9 to 23 μM [6], but this can increase several fold as a result of inflammation or immunological response [20].

Factors that have been associated with variations in drug-AAG binding include AAG concentration, AAG polymorphisms, glycan heterogeneity, pH, and competitive binding with endogenous or xenobiotic ligands [21]. Since the concentration of AAG is an obvious factor affecting drug-AAG binding, disease states having an inflammatory and/or immune component may alter the PK parameters of a drug by changing the free fraction. Specifically, modulation of AAG serum concentrations has been shown to affect drug CL when drugs are highly bound to AAG [23-26]. In certain cases, AAG polymorphism may also have an effect on free drug levels in plasma and the resulting CL of the drug [23]. Most equilibrium constants (i.e. Kd) for drugs binding to AAG fall in the single digit micromolar to millimolar range, although in some instances nanomolar affinities have been reported [21]. Extensive reviews of AAG have been written by Israili and Dayton [21] and Fournier *et al*. [9].

OTHER CONSIDERATIONS REGARDING DRUG-PLASMA PROTEIN BINDING

It is well known that drugs can bind to plasma proteins in an enantioselective manner. As a result, enantiomeric drugs dosed as a racemate often show differential protein binding and pharmacokinetic properties for each enantiomer. In general, enantioselective plasma protein binding has been primarily associated with drugs that bind with high affinity and selectively to HSA [8, 27]. However, enantioselective binding of drugs to AAG [28-30] and lipoproteins [31] have been observed.

In some instances, drug molecules can be displaced from plasma proteins by co-administered drugs and endogenous materials such as fatty acids, bilirubin, hormones, etc. The displacement of drugs from plasma proteins may result in an increase in free drug concentration, thus potentially increasing the pharmacological effect of the displaced drug. An increase in free drug concentration in plasma may also enhance the rate of elimination due to the increased availability of drug to metabolizing enzymes in the liver. Displacement of the drug and the clinical consequences will therefore be a dynamic process and will depend on the rate of elimination, the drug's therapeutic index and the rate of biological response to free drug concentration. As a result, an increase in free drug concentration may or may not be biologically relevant. Changes in free drug concentration will be clinically relevant when the drug is administered intravenously, has a high extraction ratio and is eliminated primarily by hepatic metabolism, or, when it is a high extraction ratio drug and the liver is not the main route of systemic elimination [32]. Expressed in a different way, if the therapeutic index is large and/or the biological response is slow relative to transient changes in free drug concentration in plasma, then changes in free drug concentration will result in a new equilibrium, through distribution and elimination processes, and the PD consequence of displacement will be minimal [32]. In most instances, because of large molar excess of binding proteins in serum, the relatively large therapeutic window of marketed drugs, and hepatic clearance dependency on free drug fraction, displacement of drugs from plasma proteins have little effect clinically. In a special situation where displacement of a highly bound drug might influence the CL of concomitantly dosed drugs metabolized by the same P450 isoform, drug displacement may have a greater clinical significance [33].

Displacement or inhibition of drug binding can also be evaluated *in vitro*. The competitive binding of two drugs may be exploited to determine the protein binding site when conducting drug displacement studies. For example, an increase in free fraction with the addition of warfarin would indicate drug binding to site I of albumin, whereas an increase in free fraction with the addition of diazepam would indicate site II binding. In other instances, additives and endogenous molecules that bind to plasma proteins may displace the drug by causing changes to the binding protein's structure allosterically, thus modulating the free drug fraction. In some cases displacement of drug from albumin may be stereospecific [34].

Patient demographics and disease state may also need to be considered in assessing clinical PPB. Variables such as age, race and gender can affect protein concentrations as well as protein variants, which may influence free drug fraction. Although some studies have shown a correlation between age and protein binding (i.e. free fraction decreasing with age) [35], other studies suggest that these trends are not due to age per se but to asymptomatic disease in the aged population that increase AAG levels even though the patients are apparently healthy [36]. Age-related changes in drug-PPB have been reviewed by Grandison and Boudinot [37].

In a review by Johnson, the result of ethnic differences on drug PK was evaluated [38]. It was concluded that whereas acidic drug binding to albumin have little correlation to ethnicity, drugs binding primarily to AAG do show ethnic differences with Caucasians exhibiting a lower

free drug fraction due to higher levels of AAG [38]. It was concluded that changes in PK parameters were potentially significant across different ethnic groups only when a drug was highly protein bound to AAG.

Changes in albumin or AAG levels in various disease states may need to be considered when adjusting dose in patients. Hypoalbuminaemia is often observed in patients with liver disease, renal failure, nephrotic syndrome, hyperthyroidism, burns, malnutrition, etc. [3, 39-42]. In extreme cases, patients may have saturated PPB when following normal dosing protocols. This has been highlighted for the NSAID naproxen where patients have shown increases in free fraction from secondary hypoalbuminaemia associated with active rheumatoid arthritis [43]. AAG levels are known to vary widely during immune responses and in a number of clinical conditions such as cirrhosis, nephrotic syndrome, cancer, Chrone's disease, various states of inflammation, heart disease, renal failure, rheumatoid arthritis, obesity, stress, trauma, surgery, etc. [39, 40, 42, 44, 45].

Changes in drug binding to HSA or AAG have also been reported as a result of disease-induced variations in endogenous ligands such as billirubin and nonesterified fatty acids [39]. The extent of PPB can also change with genetic variation of albumin [46] and AAG [23] and with changes to the hererogeneity of AAG glycan structure or the degree of AAG glycan sialyation [9, 20, 21, 47].

PPB will also be affected by plasma pH variations in cases of severe acidemia and alkalemia [48]. Hinderling and Hartman calculated changes in free fraction for a range of acidic and basic drugs as a function of pH and predicted that changes in pH from 7.4 to 6.7 can result in changes of free fraction ranging from 32 to –136% [48]. Similarly, with a pH change from 7.4 to 8.0 they predicted changes ranging from 100 to -86%. Interestingly, the effect of pH change on free drug fraction could not be fully explained by compound pKa alone (i.e. the degree of compound ionization) but were thought to be due to direct pH-induced conformational changes to AAG and HSA as well [48]. The predicted changes in PPB with pH observed in severe acidemic or alkalemic are thought to be clinically relevant provided the drug in question has a narrow therapeutic index [48].

METHODS TO DETERMINE PLASMA PROTEIN BINDING

The PPB methods discussed in this review were selected based on their ability to (1) determine PPB as part of a medium-to-high throughput workflow or (2) measure compounds that exhibit very low free fraction. By choosing to limit the scope of the review in this manner, some very valuable protein binding techniques such as microdialysis, microcalorimetry, circular dichroism, fluorescence quenching, will not be covered. The commonly utilized techniques of equilibrium dialysis (ED), ultrafiltration (UF) and ultracentrifugation (UC) are, however, reviewed and discussed below. In addition to these methods, some lesser utilized techniques, such as microextraction and optical biosensors, will be discussed for their ability to measure highly protein bound drug candidates.

The most commonly utilized methods for determining plasma protein binding are ED [49], UF [50] and UC [51] and are the subject of many comprehensive reviews [40, 41, 52-54] These methods rely on the physical separation and measurement of the unbound and bound drug to calculate their relative fractions. Ultrafiltration and ED, use semipermeable membranes to separate the unbound and bound fractions with subsequent analysis of the free fraction using a suitable analytical technique (e.g. liquid chromatography/mass spectrometry). While reliable and reproducible results are routinely achieved with these methods, their utility can be limited by the physico-chemical characteristics of the compound under investigation. In particular, highly adsorptive compounds can limit the use of UF and ED due to nonspecific binding of the compound to the membrane surfaces and the apparatus [55, 56]. Poor aqueous solubility of the compound may also be problematic and may limit the use of ED due to insolubility of the compound in the dialysis buffer. In general, compounds to be analyzed by ED or UF should be soluble in the dialysis buffer at the expected free drug concentration and exhibit minimal non-specific binding during the course of the experiment. When solubility and non-specific binding have been evaluated and determined to be acceptable, then other factors such as assay resolution, ease of use and throughput, may need to be considered when deciding which technique is the most appropriate. For example, in early drug discovery a low, medium and high PPB ranking system may be all that is needed to guide compound selection and derive protein binding structural relationships; while in later development, there is a need for more definitive PPB values in order to interpret preclinical results and establish dosing protocols for clinical evaluation.

There are a variety of commercially available devices for both UF and ED that can be used for sample throughput requirements ranging from single sample analysis to 96-well plate processing. Higher throughput parallel sample processing devices for ED have recently been introduced including the 96-well Rapid Equilibrium Device (RED) from Thermo Scientific/Pierce (Rockford, Il); the 96-well Equlibrium Dialyzer from Harvard Bioscience (Holliston, MA); the 96-well Micro Equilibrium Dialysis Device from HTdialysis LLC (Gales Ferry, CT); and the 24-well Serum Binding System from BD Biosciences (San Jose, CA). With the availability of these higher throughput devices, ED has developed into an attractive method for PPB determinations during drug discovery where higher throughput is needed [57-62]. An example of the 96-well RED system from Thermo Scientific/Pierce is illustrated in Fig. (**1**).

Ultrafiltration is the simplest and fastest method for determining the free fraction of drug and is therefore a good choice for clinical therapeutic drug monitoring and clinical pharmacokinetic and pharmacodynamic studies [63-66]. Some kits are commercially available for measuring the free drug levels of phenytoin, valproic acid, carbamazepine and digoxin in clinical laboratories (Abbott Laboratories, Abbott Park, IL; Bayer Diagnostics, Tarrytown, NY; Roche Diagnostics, Indianapolis, IN; Beckman Diagnostics, Brea, CA; and Ortho Diagnostics, Rochester, NY). In addition, there are commercially available 96-well UF devices (Millipore Corp., Bedford, MA; Harvard Bioscience,

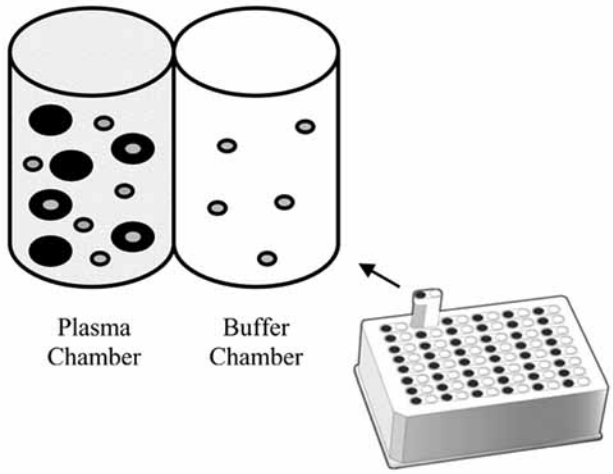

Fig. (1). Schematic diagram of a 96-well equilibrium dialysis apparatus used to determine unbound concentrations of drug. The dialysis cells are drawn after equilibrium with the black ovals representing the unbound protein and the grey ovals representing the bound and unbound compound.

Holliston, MA) which have been used for automating early drug discovery assays and pharmacokinetic studies [67, 68]. A representation of such a UF device in 96-well format is shown in Fig. (**2**).

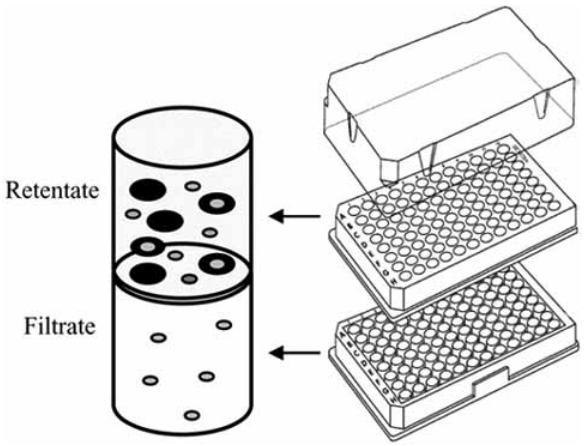

Fig. (2). Schematic diagram of a 96-well ultrafiltration apparatus used to determine unbound concentrations of drug. The ultrafiltration unit is drawn after centrifugation with the black ovals representing the unbound protein and the grey ovals representing the bound and unbound compound.

With all the separation techniques, accurate and precise measurements are dependent upon establishing and maintaining equilibrium conditions during the separation process. Temperature and pH are two parameters well known to influence equilibrium binding of small molecules to proteins [69]. A literature survey examining the effects of pH on the binding of drugs to plasma proteins noted that the relationship between the fraction unbound and the pH is linear for all of the basic drugs surveyed and for most of the acidic drugs [48]. As the pH increases, the free fraction decreases for basic drugs. No consistent trend is observed for acidic compounds. In addition, changes in albumin conformation within the physiologic pH range can modulate the binding of small molecules to the protein [14, 70-73]. Control of pH is therefore necessary when precise and accurate measurements of protein binding are performed. During blood collection and plasma separation and storage, changes from physiologic pH can occur. While freeze/thaw cycling of plasma has been shown not to influence protein binding significantly [74], it is necessary to readjust the pH to a physiologic level and control the experimental system to maintain the pH throughout the separation process [69, 74, 75]. Long-term storage of plasma is not recommended, as lipolyses can occur which can increase the free fatty acid levels in plasma [76] resulting in fatty acid-induced protein conformational changes that may influence small molecule/albumin binding [8, 77-81]. The same attention to pH and fatty acid content will need to be taken into account when preparing isolated proteins for protein binding studies [16, 18, 82]. Finally, the buffering capacity of the dialysis buffer should also be evaluated whenever maintaining the proper pH is problematic as with long incubation times and the use of sample pooling [62].

In addition to pH, temperature control is necessary to establish equilibrium conditions, as the free drug concentration will increase with increasing temperature [69, 74, 83-85]. Compound stability needs to be determined within the assay conditions, especially for ED which can require long incubation times. When spiking solvent containing stock solutions into the assay matrix, the final solvent concentration should be kept as low as possible in order not to disrupt protein binding interactions. Researchers have reported that acetonitrile can compete with certain drugs for low-affinity binding sites on HSA [86]. Others have demonstrated a decrease in protein binding after samples are spiked with acetonitrile [55]. Ethanol and other alcohols at concentrations between 0.1% to 10% have also been shown to affect warfarin stereoselective binding to albumin [87, 88]. To eliminate spiking organic solvent directly into plasma matrix, an alternative method is to prepare the appropriate standard in solvent, evaporate the samples to dryness and reconstitute in the appropriate volume of plasma taking care that quantitative solubilization is achieved during reconstitution.

Determining the free drug concentration can be technically challenging especially for highly protein bound compounds where the free drug concentration can be in the low ng/ml range [89]. Recent trends tend to suggest that a greater number of compounds advancing into drug development are highly protein bound which may in part be due to the advent of high throughput screening and combinatory chemistry, where compounds often exhibit increased *in vitro* potency and increased lipophilicity [90]. These lipophilic compounds also tend to be highly protein bound [10, 91], contributing to the need for robust and sensitive analytical techniques to determine the compound's free fraction. The most common detection methods used for free drug measurement are LC-MS/MS and, if radiolabeled compounds are available, liquid scintillation counting. LC-MS/MS is a highly selective, reproducible and sensitive technique that can detect compound in the low to sub- ng/ml range and has the advantage of being easily incorporated into an automated workflow [62, 67, 92-96]. The selectivity of LC/MS can permit increased sample throughput by using a single LC-MS/MS method to simultaneously assay up to 10

compounds in a pooled sample [62]. Mass spectrometric sensitivity is, however, dependent on the ionization efficiency of the compound [89] which can vary significantly even among structural analogs. As a result, each compound will need to be optimized independently and will have varying linear dynamic ranges and limits-of–quantification which will need to be taken into account. Liquid scintillation counting using radiolabeled compounds with high specific activity and purity, has an advantage of being compatible with direct analysis of compounds in complex matrices without extensive cleanup and having a response and dynamic range that is largely independent of the compound structure. A disadvantage of using radiolabeled compound for PPB, however, is that impurities and instability of the radiolabeled compound can lead to overestimations of the free drug levels, making it necessary to determine radiolabel purity pre- and post-separation from the bound compound [97, 98].

EQUILIBRIUM DIALYSIS

Equilibrium dialysis uses a two-chambered device with the chambers separated by a semipermeable membrane. The protein solution containing drug is placed in one chamber while buffer is placed in the opposing chamber. Unbound drug passes through the membrane, which is impervious to both bound and unbound proteins. When equilibrium is reached, the unbound drug will be at equal concentrations on both sides of the membrane while the bound drug will remain in the protein chamber. An initial set of studies are performed to determine the time necessary for the system to achieve equilibrium. Higher MW and highly bound compounds take longer to reach equilibrium [99, 100], and the equilibrium is achieved faster if the compound is added to the protein side and the system is agitated [75, 101-103]. Extensive equilibrium times can cause errors due to bacterial growth, fluid shifts, and changes in plasma pH and free fatty acid levels [77]. Once equilibrium is established, the total drug concentration is sampled from the protein side while the free drug concentration is sampled from the buffer side. The bound drug is then calculated from these measurements.

New 96-well formatted devices composed of vertical open topped cylindrical chambers have allowed for easier use and automation, producing fast reproducible results compared to the older “sandwich” systems which were prone to leaking and long incubation times. The design permits for sampling from both sides of the membrane, making recovery and nonspecific binding determinations possible. The membrane used have typical MW cut-offs ranging from 5-12 kDa.

Despite several disadvantages that can cause systematic errors, ED is the most widely used technique for determining the free concentration of drug [99]. These disadvantages include changes in initial equilibrium conditions, nonspecific binding, volume shifts, Donnan effects, and protein leakage across the membrane [40, 52, 54]. A change in the initial equilibrium concentration occurs with the movement of free compound across the membrane which establishes a new equilibrium between the bound and unbound compound [104, 105]. The difference in the initial total compound concentration and the concentration at equilibrium will increase for compounds of increasing free concentration. This becomes important for compounds where protein binding is concentration-dependent or when it is necessary to determine the initial free concentration of drug [106]. Sampling both sides of the dialysis chamber and using the total drug equilibrium concentration value to determine the percent bound simplifies the calculation but this calculated value is relevant to the post-dialysis concentration only.

Nonspecific binding to the membrane, or apparatus, can cause under-estimations in the unbound concentration. To alleviate some of these issues most apparatuses are now made with Teflon to minimize nonspecific binding. In addition, the error associated with nonspecific binding can be corrected if the calculations are made with measurements from both sides of the dialysis chamber [100, 107]. If nonspecific binding is large, however, an alternative technique that uses a different means of separation (e.g. ultracentrifugation) may be required.

Volume shifts occur when colloidal osmotic pressure forces fluid from the buffer side to the protein side of the device [108, 109]. This will dilute the protein concentration and thus alter the binding equilibrium especially for compounds exhibiting low affinity interactions. This effect is enhanced by equilibration times greater then 4 hours but can be corrected for mathematically [102, 104, 110, 111]. When the volume shift is less then 10%, the effect is considered to be negligible and is not taken into consideration when calculating the bound drug [59, 112, 113]. An alternative to these practices is to include dextran in the buffer in order to balance the osmotic pressure [60, 102].

The Donnan effect is another potential complication with membrane systems and can be especially problematic for highly ionized and low-to-moderately bound compounds. Here, an unequal distribution of diffusible ions between the two chambers is created by the nondiffusible protein ions [114-116]. This effect can be corrected for if the Donnan ratio is determined [116]. Alternatively, the effect can be minimized by increasing the concentration of electrolytes in the dialysate buffer [116, 117]. However, care should be exercised when increasing the concentration of certain electrolytes since some ions have been shown to competitively displace compounds and alter protein binding [118-120].

Finally, if the integrity of the membrane system is compromised and protein leakage occurs across the membrane, significant overestimations in the free fraction can occur especially for highly protein bound compounds and when extended equilibrium incubation times are utilized. Bower *et al.* estimated that a 9% error for a 90% bound compound would occur with 1% protein leakage [100]. The prevalence of protein leakage across membrane systems has been minimized by the introduction of the commercially available RED system which typically reaches equilibrium within 4 hours.

ULTRAFILTRATION

Similar to ED, UF uses a two chambered device that is separated by a semipermeable filter membrane. The drug protein solution is placed in the upper chamber and positive pressure or, more commonly, centrifugation (approximately 2,000 *xg*) is used to move the unbound drug from the upper

chamber into the lower chamber. The free drug concentration in the lower chamber and the total drug concentration prior to UF are then used to determine the extent of protein binding.

This technique offers several advantages over ED in that it is rapid and technically less challenging then ED. Compared to ED this method reduces errors caused by lipolysis, protein dilution and protein leakage across the membrane that may occur during extended dialysis times. As a result, UF is often used at the drug discovery stage in order to rank-order a number of compounds based on plasma protein binding. In addition, the simplicity of the UF system reduces errors caused by competing substances in a buffering system that may be used with ED.

One common misconception about UF is that the equilibrium is altered during the filtration process, with some recommending the collection of <10% of the ultrafiltrate so as to remain as close to the initial equilibrium condition as possible. In fact, the binding equilibrium does not change during UF [99, 100, 121-123], even with a two-fold concentration change of the proteins [121, 123].

The biggest disadvantage in using the UF technique is nonspecific binding of free drug to the filter membrane or collection chamber which can result in an under-estimation of the free drug concentration [121, 124-126]. This issue is becoming increasingly important as more lipophilic drug candidates are being generated by the pharmaceutical industry. Nonspecific binding can be determined by performing UF with compound in buffer or control ultrafiltrate. For this type of experiment, the compound is assayed at a concentration close to the predicted unbound concentration where losses due to nonspecific binding can be significant and the measurement is more applicable to the experimental conditions expected in plasma. Alternatively, mass balance experiments, where compound recovery is determined, can indicate the possibility of nonspecific binding. Some researchers have reported a reduction in nonspecific binding by using membranes made out of alternative materials like polysulphone [127]; while others report no substantial improvement [128]. Preconditioning the filters with serum ultafiltrate has also been attempted with no appreciable reduction in nonspecific binding [126, 129]. Some success in reducing nonspecific binding was reported by Lee *et al*. by pretreating the filter membranes with Tween-80 for neutral or acidic compounds, or with benzalkonium chloride for basic compounds [128]. Using this membrane pretreatment method in combination with a mathematical correction for non-specific binding, similar results were obtained for UF and ED as long as nonspecific binding was <50%. Other researchers have used various methods to minimize nonspecific binding of drug to the collection chamber. For example, Taylor and Harker modified the standard UF method for corticosteroid binding by including a second filtering step whereby control plasma retentate was used to mix with the previously collected filtrate to prevent binding of corticosteroids to the filtrate collection chambers [130]. This had the added benefit of producing a retentate sample that allowed for mass balance determinations. Others have used 0.1% Tween-20 to condition pipette tips and ultrafiltrate collection chambers [63] to minimize adsorptive losses.

Another reported disadvantage of UF is molecular sieving, whereby the plasma water and the free compound do not pass through the ultrafiltrate membrane at the same rate. This can result in an under-representation of the free compound in the filtrate as the water molecules pass through the membrane more quickly than the compound. Molecular sieving is reported to increase with increasing molecular size of the compound [99] and increasing pressure [121, 131].

Like ED, potential errors associated with UF are possible due to Donnan effects and protein leakage [99, 100, 121, 132]. In addition, the pH and temperature are more difficult to control during the UF process although temperature variation during centrifugation is reduced with the use of a temperature controlled centrifuge and single samples or 96-well UF devices with lids are available to maintain the pH and reduce evaporation. Even with lids, however, evaporation issues have been reported which were partly compensated for by using a lower temperature during centrifugation [67].

ULTRACENTRIFUGATION

Unlike the membrane techniques of ED and UF, UC uses the application of high gravitational force (625,000g) to separate the free from the bound compound. After centrifugation, the bound compound associated with high density plasma macromolecules like albumin, alpha-1 acid glycoprotein and other plasma proteins and lipoproteins sediment to the bottom of the tube while very low density lipoproteins and chylomicrons float to the surface of the tube Fig. (**3**). The concentration of the free compound is determined by sampling the area just below the lipid layer while the total compound concentration is determined prior to centrifugation. The bound compound can then be calculated from these measurements.

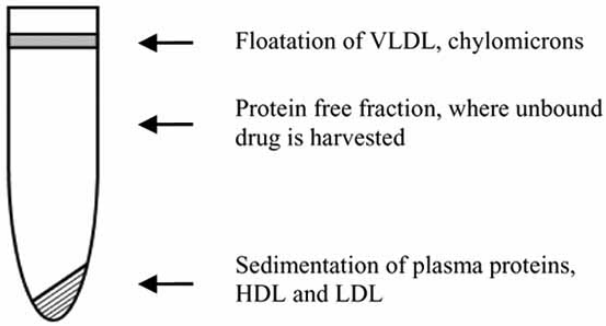

Fig. (3). Schematic diagram of a fractionated plasma sample after ultracentrifugation used to determine unbound plasma drug concentration. The upper grey band represents the lipoprotein fraction while the lower hashed area represents the sedimented plasma proteins, LDL and HDL. Unbound compound is harvested below the upper lipid layer within the protein-free fraction.

The equipment used for UC is expensive in contrast to the equipment used for ED and UF. The newer bench-top ultracentrifuges are more convenient compared to the older style preparative machines and provide several distinct advantages with shorter run times, smaller sample size and temperature control. The rotor design and tube configurations for the modern equipment allow for run times as short as 4 hours while using sample sizes of less then a

milliliter. The time (*t*) required for complete separation can be calculated from the clearing factor *k* for a specific rotor with its unique radial distance maximum and minimum values (r_{max}, r_{min} respectively), the rotor speed, given as revolutions per minute (*rpm*), and the Svedberg sedimentation coefficients (*S*) for the limiting major plasma proteins and lipoproteins (Equations 3 and 4). Here, time is proportional to the clearing factor and inversely proportional to the sedimentation coefficients.

$$k = \frac{2.53\times10^{11}\ln\left(r_{\max}/r_{\min}\right)}{rpm^2} \quad (3)$$

$$t = k/S \quad (4)$$

The newer equipment also allows for a physiologic temperature to be maintained throughout the run; a feature missing from some older equipment. However, physiologic conditions are not preserved with respect to pH as a starting pH of 7.4 will increase to 7.8-8.0 during the centrifugation process [133].

A retrospective examination of the literature indicates that the experimental run times used by some laboratories were insufficient to allow for complete separation of the plasma constituents and in addition to the absence of temperature controlled centrifugation has lead to some misinterpretations of their results. Some authors have compared results from UC with the membrane techniques even though the effects of temperature and pH on their systems were not taken into consideration [134]. Indeed, many authors have noted contamination by proteins or lipoproteins within the protein free layer [55, 113, 133-135]. Nakai *et al.* identified the contaminating proteins and estimated that the contaminating proteins produce an error resulting in an overestimation of unbound fraction by 13% for drugs that are 1% unbound with this error becoming much more significant for drugs that are < 1% unbound. Therefore, for highly bound drugs, it is critical to apply the appropriate centrifugation parameters to produce protein free material. Additional errors may occur at the time of sample harvesting if the upper lipid layer is disrupted or ignored. Some researchers harvest by slicing the tube below the lipid layer [134]; while others remove the lipid layer by aspiration before harvesting [55]. The harvesting technique becomes of greater importance for drugs that bind lipoproteins as mixing of the upper layer lipid layer may increase the apparent unbound drug concentration.

For highly absorptive compounds, UC is a good alternative to the membrane techniques for there are fewer nonspecific binding issues to the ultracentrifuge tubes as compared to binding to dialysis or ultrafiltrate membranes [55, 99]. UC also requires less time for assay validation in that there is no need to establish a time to equilibrium as with equilibrium dialysis, and there are fewer issues with the Donnan effect [136].

A disadvantage not overcome by modern equipment is the relatively small number of samples that can be processed at one time; restricting this technique to low throughput applications. There are other reported disadvantages to the centrifugation method suggesting that it might not be a "gold standard" or definitive technique. Since a number of references make note of an apparent complexity and difficulty of separating free and bound molecules, it is worth discussing some of the factors that contribute to the confusion and apparent issues with the technique in hope that the reader can avoid the various pitfalls in their experiments and properly utilize the technology for more definitive binding measurements.

As noted above, selection of spin time is a critical parameter in sedimentation methods; such that particular care should be taken when estimating transit times to pellet the drug binding plasma proteins, based on calculations utilizing apparent *S* values (Equ.. 4). The hydrodynamic parameters typically given in the literature are for standard conditions of dilute solution at 20°C and in water ($S_{20,w}$), however, serum and plasma solutions are "crowded" environments. This significant difference primarily arises from HSA being present at a concentration of several hundred micromolar, but other plasma components also contribute and the corresponding impact is a much higher solution viscosity and density than water. The various crowding factors, along with transport anomalies unique to biological fluids, such as the transit of very large floating particles like LDL and VLDL to the top of the tube, have a dramatic impact on the sedimenting and diffusive mobility of molecules being centrifuged [137-139]. There are other, rather significant, hydrodynamic anomalies that arise in crowded solutions and contribute to the complexities as well, such as the Johnston-Ogston effects [140], but unfortunately a discussion of all the various factors is beyond the scope of this review; however, the reader can find good treatments of the topics in standard biophysical chemistry texts [141, 142]. The overall effect of these various transport impediments in serum/plasma solutions causes the apparent *S* values for molecules that sediment with or faster than the dominant albumin to be lower by ~2 *S* units; interestingly, molecules that sediment more slowly than albumin are slowed by ~0.2 S (Tom Laue, personal communication). Indeed, the $S_{20,w}$ value for HSA typically is reported as being ~ 4.6S, but the apparent *S* value for HSA recovered by our laboratory from applying analytical ultracentrifugation analysis to serum solutions is ~ 2.6 S (unpublished data). Additionally, reported *S* values are determined by analytical ultracentrifugation methods and are most valid when applied to experiments using sector shaped cells; wherein this geometry minimizes anomalies that effect molecular flow, such as turbulence from collisions with the container wall [141]. The typical rotor and tube configurations of bench top and preparative ultracentrifuges used for plasma binding studies are wrought with turbulent, non-ideal flow for sedimenting and diffusing molecules; resulting in a poorer separation and striation of molecules and ultimately a longer transit time to pellet formation. With these various factors in mind, we have found success in calculating spin times based on the sedimentation of a 2S particle as the limiting component. Additionally, the increased mobility that comes from a run temperature of 37°C appears to help compensate for some of the non-ideal factors impeding transit, providing us ample time to pellet and pack proteins of interest while maintaining work-day friendly run times and nominal sedimentation of the unbound compound.

A frequently cited disadvantage of ultracentrifugation is a supposed alteration of the binding equilibrium during the

centrifugation process, wherein the separation of different sized species is conjectured to disrupt mass action relationships [99, 113, 133, 134]. The various discussions revolve around suppositional deviations from the well renowned Gilbert-Jenkins theory (GJT) for interacting and sedimenting systems [143-145]. In the 1950s, Gilbert, Jenkins and co-workers solved the transport equations for two-component rapidly interacting systems undergoing sedimentation in a rectangular cell, but with the limiting assumptions of constant force, no diffusion, and constant concentration. However, since gravitational force along the tube is not constant but rather a function of radial position, molecules do undergo diffusive motions during sedimentation, and the concentrations of components evolve with radial position and time have been misconstrued by many authors as a failure of GJT to adequately describe centrifugation and support the use of the method for thermodynamic studies. However, recent and more extensive numerical simulations and empirical studies have given us a firm basis for a broad applicability of GJT. Data from numerous reacting-sedimenting-diffusing systems, including rapidly and slowly interacting small molecule-protein and protein-protein reactions, are all well predicted and analyzed and that centrifugation is a completely valid method for thermodynamic studies [146-148]. Indeed, the applicability of GJT has been illustrated for a plasma protein binding system through a detailed study in which the authors determined the ratio of total phenytoin and total protein simultaneously [149].

Extensive sedimentation of free drug during the experimental time frame has been considered by many over the past several decades as a significant source of error with the centrifugation methods; since the measured quantity of free-drug potentially will depend on where along the tube the sample is taken. Ultimately this could limit the use of the technique to very slowly sedimenting compounds, such as those with a molecular weight less than ~400 [55, 99, 135, 149, 150]. It is expected that in a high gravitational field, low molecular weight compounds will undergo some sedimentation. However, the question is to what extent this occurs during the time course of a typical experiment, since small molecules have low sedimentation coefficients and high diffusion coefficients. In the extreme case where samples are spun at high speed for a long time, on the order of days, a time-invariant concentration gradient develops as the flux of sedimenting molecules is exactly balanced by the flux of diffusing molecules at each point in the cell, forming a smooth exponential gradient. Under the conditions used for plasma protein binding work, no "pelleting" of small molecules should occur per se, unless the relative increases in concentration towards the end of the tube leads to a loss of solubility or nucleation of aggregates [151]. The overall inclination of small molecules to sediment and form appreciable gradients is driven, to different extents, by various compositional and energetic factors; for example, the steepness of the equilibrium gradient increases with decreasing density and with increasing mass, rotor speed and radial distance by favoring sedimentation over diffusion. Run temperature impacts the gradient steepness in a complex manner; for instance, increasing the temperature increases sedimentation rates through decreasing the solution viscosity and density, but heat also increases the thermal activity of molecules and coupled with the lowered viscosity enhances diffusion, which ultimately flattens a gradient [152]. Most import to this discussion is transit time to formation of the gradient and, in general, this is relatively long for plasma solutions since the initially high viscosity and density, coupled with the various non-ideal flows, impede sedimentation. Also, transit time largely is dependent on the radial length of the tube, in that time to equilibrium approximately depends on the square of the height of the solution column. Given the relatively large volumes and short run times employed in plasma protein binding studies, only a slight net sedimentation should occur in a typical experiment [153]. Additionally, once the centrifuge is stopped, and there is no longer an applied force, significant back diffusion should occur during the minutes before sample removal from the nearly protein-depleted, less viscous solution; plus, the slight "jostling" of the rotor and tubes upon sample removal potentially could help disrupt a remaining unstable gradient. Some laboratories have shown that significant drug gradients can be established, but these experiments were carried out in aqueous solutions or solutions of methylcellulose in order to mimic plasma viscosity [99, 149]. However, they neglected the contributions of other first-principle based components like solution density, temperature, centrifugal force and time, and the repulsive, attractive and exclusion forces that significantly influence movement of molecules in a flowing, crowded environment. Additionally, compounds were assayed at high loading concentrations where the initial solubility was not determined. Interestingly, when plasma ultrafiltrate was used in a similar set of experiments for a saturated solution of tirilazad, no gradient was established [55]. A definitive analysis of the problem requires a complete system that explores both the gravitational potential energy and the electrochemical potential energy of the small molecule relative to the total internal energy of the system. Given the differences in size and charge between drug molecules, a proper analysis of the problem will require a large sampling of compounds and conditions before any theoretical or empirically generalized conclusions can be drawn [154, 155].

As a rule-of-thumb, the faster the rotor speed the better the separation and the shorter the run time that is required to complete the experiment. Moreover, minimizing run time to that of clearing the smallest drug binding protein and using a biologically relevant temperature of 37°C appears to help minimize some drug sedimentation issues and provides the most thermodynamically meaningful information.

EXTRACTION AND PARTITIONING TECHNIQUES

Solid-Phase Microextraction (SPME)

There are a number of methods that incorporate the extraction of a small amount of free drug from plasma onto the hydrophobic coating of a solid-phase support. We've chosen here to describe them under the common heading of microextraction techniques although they are unique in the specific extraction devices and coatings that have been employed. The microextraction technique is attractive because the free drug is never partitioned into a solution that is void of protein so very hydrophobic molecules that have limited water solubility may be analyzed by this

methodology. In general, the technique involves adding an extraction material coated onto a solid support such as beads or fibers into a plasma, or protein containing buffer system, and binding the free drug onto the hydrophobic coating. The extraction material is then removed from the protein solution and the portion of free drug that had absorbed to the extraction surface is measured. This value is then used to calculate the free drug level in the original sample. Although the applications of microextraction to drug-protein binding have only been implemented in a low throughput manner, it appears that the methods could be compatible with high throughput workflows if coupled to automation.

An example of this technique was published by Minagawa *et al*. where glass beads coated with polydimethylsiloxane were used for this purpose [156]. In this work, the researchers demonstrated that the unbound level of a [^{3}H]-prostaglandin I_2 analog was successfully determined using SPME even though this compound could not be analyzed successfully by other commonly used protein binding techniques (e.g. ED, UF, UC or gel filtration) due to losses of the compound to membranes and container surfaces. The accuracy of the method was validated against UF by measuring free diazepam in serum where the percent binding values obtained by the two techniques were essentially identical. Like other SPME methodologies, a key aspect of the method is to first determine a standard curve for drug binding to the polydimethylsiloxane glass beads (PDMS-GB) in a suitable buffer system. In this case, extraction of diazepam was shown to be linear up to 500 ng/mL with approximately 24% of the drug being constantly absorbed onto the PDMS-GB (standard curve equation Y = 0.236X + 0.554 ng/mL r = 0.997). Linearity was also shown for the prostagladin I_2 analog (r = 0.999) with extraction efficiency of approximately 82% having a linear standard curve over the concentration range of 0.5 to 10 ng/mL. The methodology was successfully utilized to measure percent bound levels with high precision for the prostaglandin I_2 analog in essential fatty acid-free HSA, human serum and dog serum. Values ranged from 85% bound for HSA, to 94 and 98 % bound for dog and human serum, respectively. The percent bound levels were also measured for the desmethyl metabolite of the prostaglandin I_2 analog with high precision in these same matrices as well as rabbit and rat serum.

In a series of works by Musteata *et al*., solid-phase microextration (SPME) has been used to measure free drug *in vivo* [157], in liposomal formulations [158] or as a tool for *in vitro* free drug measurements [159, 160]. The utility of the technique for *in vitro* PPB determinations was demonstrated by accurately measuring free drug levels for ibuprofen, warfarin, verapamil, propranolol and caffeine in diluted human plasma [159] Two different fiber coatings, liquid polydimethylsiloxane and solid polypyrrole, were evaluated giving similar PPB results with different experimental optima including equilibration times. In another work by Musteata *et al*., microextraction phases based on polyacrylonitrile were developed and extensively evaluated for their utility to determine free drug levels in plasma [160]. The solid-phase extraction devices utilized in this study incorporated commonly used reversed-phase chromatography particles (e.g. C18, RP-amide and HS-F5) dispersed in polyacrylonitrile and showed that these devices were effective in the extraction of free drug while minimizing nonspecific absorption of proteins. Other microextraction methods have been used for the determination of diazepam binding to HSA using a poly(dimethylsiloxane) fiber SPME device with gas chromatographic detection [161].

Microextration has also been utilized in a nonequilibrium extraction mode in which the extraction device is withdrawn from the solution before equilibrium is established between free drug and drug bound to the extraction device surface. This technique has been termed negligible depletion solid-phase microextration (nd-SPME) and has been used to determine protein binding of [^{3}H]-estradiol by Heringa *et al*. [162]. This technique has been reviewed by Heringa and Hermens in which the advantages and practical limitations of the technique are discussed [163].

In addition to solid-phase microextraction, Fu *et al*. have demonstrated that liquid-phase microextraction is also feasible to measure free drug fraction [164]. This is accomplished by using a small volume of n-octanol (e.g. 25 μL) contained in a hollow polypropylene fiber membrane immersed in an aqueous solution of albumin or serum containing drug. The free drug partitions into the organic phase which is contained in the hollow fiber by hydrophobic forces. After equilibration, the amount of drug in the organic phase is measured and compared with a calibration curve to obtain free drug concentration. Equilibrium was established usually within 40 minutes for the model systems investigated.

Solid-Supported Lipid Membranes

Recently, a promising variation of the microextraction methodology based on solid-supported lipid membranes was demonstrated by Schumacher *et al*. [165]. In this paper the authors presented a detailed study on the determination of highly protein bound compounds that is both accurate and precise and is compatible with high-throughput analysis. Eight compounds with a wide range of lipophilicities (log P = 1.9 to 5.6) and free fraction (fu = 0.018 – 35 %) were used to test the methodology. Results were identical to those obtained using ED, UF or erythrocyte partitioning.

The method is performed by first extracting compound from a buffer system using porous silica beads noncovalently coated with a layer of egg yolk phosphatidylcholine. The beads are commercially available from Nimbus Biotechnologie GmbH (Leipzig, Germany) under the brand name Transil®. The volume of beads and drug concentrations used in the initial buffer partitioning experiment are selected based on the expected free fraction and drug lipophilicity obtained through *in silico* calculations. A membrane affinity value is then calculated based on the partitioning of drug between buffer and the bead's surface. The affinity value obtained in the buffer experiment is then used to determine optimal drug and bead concentrations to be used for drug extraction in diluted plasma. The optimal experimental conditions are usually obtained when the amount of compound in the lipid layer and the amount of compound in buffer are equal (i.e. $n_{buffer}/n_{lipid} = 1$). The volume of beads and the plasma dilution factor are adjusted accordingly, and a membrane affinity value for the compound in diluted plasma is calculated. The ratio of the two membrane affinity values is used to obtain the free fraction in diluted plasma which can then be used to calculate the free fraction in undiluted plasma using the plasma dilution factor.

High throughput analysis was performed in 96-well plates with glass inserts to minimize non-specific binding to the container walls. In cases where compounds show adsorptive losses, the authors describe a methodology to deal with this situation which entails increasing the amount of beads used in the assay (e.g. to obtain $n_{buffer}/n_{lipid} = 0.05$) and working at higher drug concentrations. Overall, the method showed excellent accuracy and precision even with highly lipophilic drugs exhibiting free fractions < 0.1% giving values essentially identical to those obtained using an erythrocyte partitioning technique [166].

Erythrocyte Partitioning

A variation on the microextraction methodology is the technique of erythrocyte (RBC) partitioning [166-168]. Like solid-phase microextraction, the RBC partitioning method allows isolation of free drug without first transitioning the sample to a buffer system or exposing the sample to large surface area membranes where losses of poorly soluble and hydrophobic molecules can occur. Instead, a known amount of red blood cells are added to a sample and the free drug is allowed to partition into the erythrocytes. In a method presented by Schuhmacher *et al.* [166], this is repeated for drug in buffer and diluted plasma. In each matrix, the drug concentration is measured before and after centrifugation to obtain total drug concentration as well as a RBC-depleted drug concentration, respectively. The amount of drug partitioning into the eyrthrocytes in each matrix is then calculated using a known hematocrit value. The erythrocyte drug concentrations are then used to calculate the partitioning coefficients of drug in buffer and in diluted plasma from which the free fraction in undiluted plasma can be calculated [166].

Because the compound must be allowed to partition into the erythrocytes the throughput of the assay is low and is not amenable to samples that may be hydrolyzed in plasma over the time course of the experiment. Also, care must be taken not to lyse a portion of the erythrocytes before they are removed from the sample by centrifugation or the calculated free levels will be incorrect. In general, this methodology is time intensive and is not considered a high throughput technique but may be useful for measurement of compounds that are highly protein bound, very lipophilic or exhibit non-specific binding to surfaces such as dialysis or UF membranes [166].

BIOSENSORS

Biomolecular Interaction Analysis Using Surface Plasmon Resonance

There are several manufacturers of optical biosensors including Biacore Inc. (GE Healthcare, Piscataway, NJ), Corning Life Sciences (Corning, NY), NeoSensors (Sedgefield, County Durham, UK) Reichert Life Sciences (Depew, NY) and Bio-Rad (Hercules, CA), but to avoid describing the intricacies of each instrument, the commercial instrument developed by Biacore® will be used to describe the essential features of the technology.

The measurement of molecular interactions in the Biacore® line of instruments is accomplished by a process known as surface plasmon resonance (SPR). The process, described in detail elsewhere [169, 170], arises when a p-polarized beam of monochromatic light is focused onto a thin metal film at an angle greater than the critical angle. Under these conditions, the light is said to undergo total internal reflection but a portion of the electromagnetic radiation known as the evanescent wave is able to extend up to one wavelength into the interfacial boundary and interact with the surface plasmons in a resonant manner. When this interaction occurs, the intensity of reflected light decreases and can be detected using a light sensitive detector. Because the SPR process occurs at the interfacial boundary, changes in refractive index of the material extending up to 1 μm from the metal/liquid interface will change the angle of resonance [169]. Monitoring changes in the resonance angle can then be used to detect slight changes in the refractive index in the buffer on the opposite (non-illuminated) side of the metal film [169]. During a binding event between a ligand and an immobilized target molecule, a change in refractive index will occur and be detected as a change in the SPR angle. The changes are recorded in real time and displayed in a sensorgram which is a plot of response units (RU) *vs* time from which association and dissociation constants of the binding event can be calculated. Because it is the refractive index changes that are detected, labeling of the ligand or capturing molecule is unnecessary. This is a major advantage of SPR-based measurements. An example of a hypothetical sensorgram indicating ligand-target association, equilibrium and dissociation phases of the interaction is shown in Fig. (**4A**).

Another important feature of the Biacore® instrument is the ability to immobilize a target molecule onto the surface of a microchannel fabricated on a Biosensor chip. Nonspecific interactions to the sensor chip surface are minimized by a layer of dextran that can be chemically modified for target molecule immobilization. This layer of dextran minimizes nonspecific binding to the surface and acts as a flexible tether to which molecules can be covalently linked to the sensor chip surface thus minimizing steric interferences that may result if a flat rigid binding surface were employed. A schematic representation of this surface is shown in Fig. (**4B**).

As stated above the refractive index change, and hence the magnitude of the signal reported in the form of a sensorgram, is proportional to the mass of material that binds to the sensorchip surface. The immobilized target molecule can then be exposed to potential binding partners by way of accurate volume injection which is delivered to the surface using precisely controlled microfluidics. An injected sample is then transported to the immobilized target molecule where ligands bind to the surface and cause an increase in response in the sensorgram. This area of the curve represents the binding association part of the curve as depicted in Fig. (**4A**) from which an association constant can be determined. As the injected material flows over the immobilized target molecule, an equilibrium is established between the associative and dissociative processes between ligand and immobilized target molecule which is a measure of the equilibrium constant at fixed ligand and target concentrations. As the injection plug moves out of the microchannel the solution no longer contains ligand and the sensogram begins to show a drop in response units representing the pure

disssociative process from which the target/ligand dissociation constant may be determined (Fig. **4A**). Response due to bulk refractive index changes due to inconsistencies between running buffer and injection buffer are minimized by matching the solution compositions and also by subtracting responses from a control flowcell. The control flowcell is also used to correct for any nonspecific binding the ligand may have to the microchannel surface.

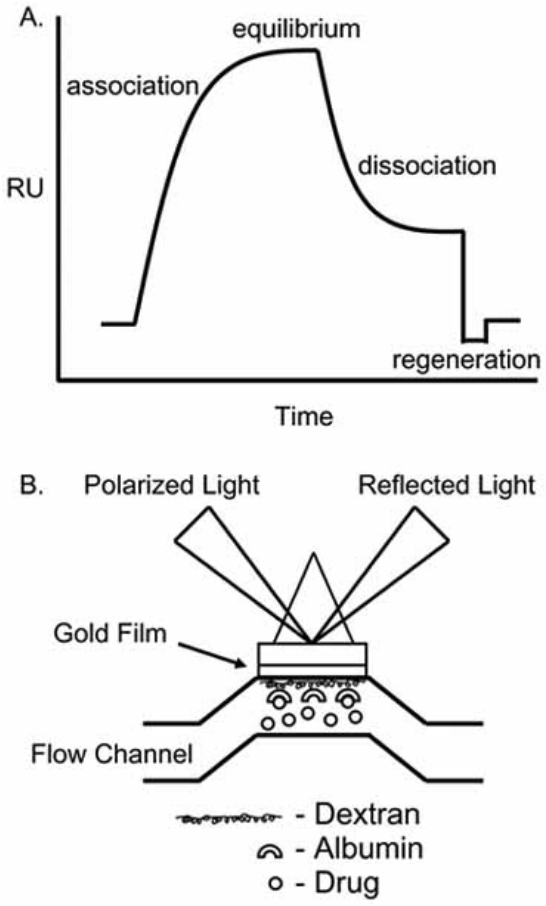

Fig. (4). A diagram of (**A**) an example of data generated from a Biacore® SPR biosensor depicting events occurring when a solution of drug is passed over an immobilized specific binding partner, as well as, the surface regeneration step, and (**B**) a schematic of the Biacore® SPR biosensor device illustrating the essential detection components of the instrument and the binding of a drug to immobilized albumin.

The scale for the response units as related to the amount of material binding to the sensor chip surface for proteins is about 1000 RU = 1 ng/mm^2. For a large molecule interaction, such as an antibody binding to an immobilized ligand, the response per mole of antibody binding is quite large (i.e. 1000 RU = 6.7 fmole/mm^2) and accurate measurements of binding kinetics can be achieved at very low concentrations. In contrast, when the binding ligand is a small molecule, as is the case for a drug binding to immobilize albumin, the RU change per binding event is small and the binding constants has been historically difficult to measure. However, using the most recent commercially available instrumentation with refined hardware and data processing capabilities, as well as improve experimental design, the accurate detection of a small MW species binding to an immobilized protein is now possible [171]. For example, Myska and Rich showed that the percent bound of warfarin to immobilized HSA, as determined by SPR biosensor analysis, could be obtained by fitting equilibrium binding data to a binding isotherm for multiple warfarin concentrations injected over an HSA surface [171]. The value obtained (i.e. 97.5% bound) was in good agreement with previously reported values obtained by ED. Rich *et al*. expanded on this original worked and showed that the Biacore® technology could be used to determine percent binding of a number of drugs in a high throughput manner using the same technique of global fitting of equilibrium dissociation constants determined at multiple concentrations or at one concentration when warfarin was analyzed on the same immobilized HSA surface and fitted simultaneously with the test compound [172]. Using this protocol, the authors estimate that 400 to 500 compounds could be analyzed per week per instrument. The equilibrium dissociation constants (Kd) for both the high and low affinity binding of warfarin to different sites on albumin could be determined, Kd = 3.7 mM and 273 mM, respectively [172]. The analysis of ten drugs binding to immobilized albumin gave percent-bound measurements that were consistent with previously reported values using other techniques [172]. Continuing the development of this methodology, Day and Myska analyzed the binding of 12 drugs with a wide range of molecular weights and affinities to various preparations of HSA using Biacore® [173]. Protocols were established to optimize albumin immobilization and drug binding. Compounds were determined to have equilibrium affinities ranging from K_D = 2.5 mM to 190 mM. These data were then fitted using either a one or two-binding site model to obtain proper fitting typically over a 10,000-fold drug concentration range. Percent bound values for each drug where than calculated from the K_D obtained for the high affinity binding site interaction. The calculated values correlated to those obtained by other PPB techniques and ranged from >99.9 % bound for dicumarol to approximately 22% bound for quinine. Notably, greater precision, accuracy and binding discrimination was obtained as drug binding increased – thus highlighting a potential benefit of this technique for highly protein bound drugs [173]. The authors also presented data on the binding of these drugs to albumin from multiple species and demonstrated the ability of SPR biosensor technology to determine the primary albumin binding site using drug displacement experiments. In a similar work reported earlier, Frostell-Karlsson *et al*. demonstrated the ability of the SPR biosensor to rank order drugs binding to HSA and AAG as high, medium or low using a single drug concentration [174]. The throughput of this single concentration ranking method was estimated to be about 100 compounds in a 24 hour period. The Biacore® technology has also been utilized to determine PPB for anti-tumor and anti-AIDS drugs [175], free warfarin in plasma ultrafiltrate [176] and for warfarin enantiomers binding to albumin [177]. Other biosensor devices such as resonance mirror [178, 179], piezoelectric quart crystal [180] and capacitive sensing [181] biosensor techniques have also been employed for PPB determinations.

SPECIAL CONSIDERATIONS

A wide range of experimental parameters can affect the measurement of PPB. Therefore experimental variables such drug concentration, protein concentration, pH, temperature, compound stability, drug displacement, etc. should be evaluated over the range of expected values. An example of such variability was reported by Paxton and Calder for the drug propranolol where slight changes in pH or temperature gave significant changes in PPB [69]. In this same study, propranolol showed no change in bound fraction with drug concentration over the range 10-500 ng/mL but was shown to be linearly correlated with serum AAG concentrations in diseased patients.

Additionally, researchers may need to investigate the PPB of active drug metabolites. It has been shown that the rapidly cleared Angiotensin II receptor antagonist drug, tasosartan, has a prolonged drug action primarily due to the significantly tighter protein binding of its active enol metabolite, enoltasosartan [182]. In this study [182], the terminal half-life of enoltasosartan was estimated to be at least eight-fold longer than the parent drug. *In vitro* studies showed that the two active species have similar receptor binding. However, in the presence of plasma tasosartan showed only a slight change for the observed IC50 value whereas the IC50 value for enoltasosartan was increased by almost 1000-fold, indicating a much tighter binding between plasma proteins and the enol metabolite.

In some instances the experimental parameters affecting PPB may not be totally understood or obvious. Additives to the dosing solutions of intraveneously administered drugs may also need to be considered. In a study by Sparreboom, *et al*., researchers showed that RBC partitioning of paclitaxel is influence by the i.v. vehicle component Cremophor EL (CrEL) thus altering free levels of drug [183]. In this same study it was shown that with i.v. dosing of paclitaxel, the drug preferentially partitions into CrEL micelles thus decreasing free drug levels available for biological activity and for RBC partitioning and transport. The authors proposed that these changes in blood distribution are responsible for nonlinear exposure (e.g. plasma Cmax and AUC) of drug with dose escalation studies observed in animals and humans [112, 183].

In a study of apparent protein binding of quinidine in rabbit plasma, Guentert and Oie showed that the measured free quinidine fraction was influenced by heparin concentration as well as the type of buffer used to determine free fraction by ED [75]. It was shown that at a heparin concentration of 20 U/mL the free quinidine was elevated compared to samples void of heparin. When the concentration of heparin was diluted to 5 U/mL, protein binding was unchanged from that observed for heparin-free samples. The increase in free quinidine level was also observed *in vivo* when heparin was injected at a level of 450 U/kg. In this same study, it was demonstrated that the degree of protein binding was also influenced by certain dialysis buffers due to displacement of quinidine from plasma proteins by chloride ion contained in the buffer.

In a study designed to explain observations linking increased mortality in critically-ill patients to albumin infusions, Olsen *et al*. compared the protein binding of pharmaceutical-grade albumin, often used to boost the osmotic pressure during surgery and in critically ill patients, to that of native human serum and non-pharmaceutical-grade commercial albumin [184]. The authors showed that the pharmaceutical grade albumin displayed significantly lower protein binding than either the human serum or the non-pharmaceutical-grade commercial albumin. In the case of naproxen, the pharmaceutical-grade albumin showed a 40-fold increase in free fraction whereas warfarin and digitoxin showed increases of 5 and 2-fold, respectively. It was concluded that the differences in drug binding were most likely caused by stabilizers (caprylic acid and N-acetyl-DL-tryptophan) added to the pharmaceutical-grade albumin before pasteurization since similar protein binding measurements were obtained after removal of the stabilizers by charcoal adsorption.

By presenting these examples we hope to emphasize the number of hidden factors that may contribute to PPB changes *in vivo* as well as during free drug determinations. To this end, *in vivo* consideration of drug displacement by other drugs or high level metabolites may need to be evaluated. In the special case of intravenously administered drugs, the effect that formulation additives may have on PPB should be investigated. In addition to normally controlled variables, such as temperature and pH, the effect of some not so obvious parameters, such as ionic strength and buffer composition, may need to be considered.

COMMENTS REGARDING PPB MEASUREMENTS

Factors effecting the PPB measurement such as compound solubility, temperature, volume shifts, organic content, non-specific binding, etc. should be understood and tightly controlled or eliminated during the analysis. Certain methods such as ED, UF, UC, and the partitioning and extraction methods describe above, are amenable to measuring free drug in plasma whereas some other techniques such as Biacore®, measure protein binding using individual proteins. Therefore, when selecting the method to measure free drug fraction, it's important to understand what information is needed from the PPB measurement and for what purpose. For example, if free drug levels are needed to develop PK/PD models in multiple species then, obviously, a technique that is amenable to measuring free drug levels in plasma (or diluted plasma) is most applicable. However, if a compound is known to predominantly bind to albumin, or if the objective of the measurement is to identify binding site or rank order drug candidates, then other techniques that measure drug binding to a single protein may be appropriate. It should be noted that when drug protein binding is determined to a single protein, the degree of protein binding will in many instances be underestimated compared to the same measurement conducted in plasma. Hence, the free fraction determined in a single protein experiment will actually be higher than that observed in plasma due to the potential of a drug binding to multiple plasma proteins.

Compounds that have limited water solubility are the most difficult to accurately analyze. To make the analytical challenges even more stressful, these compounds are often the most highly protein bound. In these circumstances, special attention should be paid to compound adsorption to surfaces and compound precipitation when the analyte is

removed from plasma and exchanged into a buffer system. Under these circumstances, a method that minimizes free compound exposure to surfaces and aqueous buffer systems void of binding proteins (e.g. solid-supported membrane extraction, erythrocyte partitioning or UC), would conceivably be the method of choice for these difficult compounds.

Free drug concentration can be a function of total drug concentration. Typically, the measurement of free drug should be done at low concentration and over a wide concentration range to determine if protein binding saturation occurs. The therapeutic range of the drug, or drug candidate, should also be considered. As stated previously, saturation of protein binding often occurs at levels of drug that are well in excess of therapeutic doses and are most often clinically irrelevant. However, this saturation information is necessary during the drug development stage to determine proper clinical safety margins from toxicokinetic studies performed in preclinical toxicological animal models. This is especially important if doses used in the toxicokinetic studies are high enough to saturate protein binding.

Lastly, it is well known that the most critical need for accurate free drug level measurement is when protein binding is high. At a protein binding level of 99.8 % an absolute error of 0.1 % in the measurement of free drug will translate to a +/- 50 % uncertainty in free drug levels. If the therapeutic window of the drug is narrow, this uncertainty may be a major concern in predicting a safe dose for clinical evaluation. In these instances, experimental variability will need to be understood and tightly controlled. In addition, the detection method, such as LC/MS/MS or radioactive decay measurements, must be sensitive, specific, accurate and precise enough to measure changes in free drug levels at very low free drug concentrations.

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ABBREVIATIONS

HSA	=	Human serum albumin
AAG	=	α-1-Acid glycoprotein
ED	=	Equilibrium dialysis
UF	=	Ultrafiltration
UC	=	Ultracentrifugation
V	=	Volume of distribution
CL	=	Clearance
PPB	=	Plasma protein binding
fu	=	Fraction unbound or free drug fraction
RBC	=	Red blood cell
PK	=	Pharmacokinetics
PD	=	Pharmacodynamics
MW	=	Molecular weight

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