12 The Significance and Determination of Plasma Protein Binding

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12.1 INTRODUCTION

Investigations pertaining to profiling the pharmacokinetics (PK) of a drug, require that a drug dose be administered either intra- or extravascularly, followed by measurement of blood, plasma, or serum drug concentrations to follow and mathematically model the fate of the drug. Following drug administration, the drug finds itself directly in the systemic circulation (intravascular dosing) or has to reach the systemic circulation via the process of absorption (extra-vascular administration). The drug then undergoes the process of distribution (defined as the reversible transfer of drug from and to the systemic circulation) that results in the drug reaching the sites of action, the sites of adverse effects, and the organs of elimination. Biotransformation or excretion by the organs of elimination subsequently leads to the loss of drug from the system and the termination of biological activity [1,2].

The drug in the systemic circulation can interact with many of the components of the blood and likewise may also interact with non-target components in the tissues. These interactions generally involve reversible binding to blood or tissue proteins mediated by noncovalent interactions that include ionic, electrostatic, hydrophobic, and van der Waals forces [1,3]. The consequence of these interactions is increase in the apparent

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molecular mass and molecular size of the bound drug. This leads to the inability of the complex (or drug) to permeate across membranes by simple diffusion or by glomerular filtration thereby affecting the rate and extent of drug distribution and renal excretion. Furthermore, these interactions also result in an inability to enter the active site of the target and/or drug-metabolizing enzymes thus impacting the pharmacological activity and/or the first-pass or systemic elimination or clearance of the drug.

The components involved in these interactions in the blood are plasma proteins and include human serum albumin (HSA), alpha acid glycoprotein (AAG), globulins (α, β, and γ), lipoproteins [including chylomicrons, very low density lipoproteins (VLDLs), low density lipoproteins (LDLs), high density lipoproteins (HDLs), and other related forms of lipoproteins), transcortin, to name a few [3]. In addition, the drug may also be sequestered in blood cells such as erythrocytes, leucocytes, and platelets. Binding of drugs can also occur to tissue proteins and result in differential nonhomogenous distribution of drug within and among tissues [1,3].

Among all of the interactions listed, the interaction with plasma proteins has arguably the maximum impact on both the PK and the pharmacodynamics of drugs, in addition to the very specific binding to the target molecule involved in drug action. HSA and AAG, in this regard, have a greater impact on plasma protein binding than other plasma components. HSA is a single polypeptide chain with a molecular mass of about 66 kDa (585 amino acids). HSA is present in plasma at concentrations as high as 50 mg/mL (about 700 μM). It is known that HSA has six different types of binding sites that have been delineated based on the binding properties. For example, type I and II sites (present up to three times on an HSA molecule) bind warfarin and diazepam, respectively. Similarly, type III site (present at six or more regions on an HSA molecule) binds phenytoin in an apparently nonsaturable manner. Some of other drugs known to bind to HSA are NSAIDs (nonsteroidal anti-inflammatory drugs), digoxin, imipramine, theophylline, caffeine, probenecid, tamoxifen, and so on [1,3,4]. Both the high concentrations in the plasma and the presence of a multitude of binding sites make HSA the most versatile of proteins that bind drugs. AAG is a single polypeptide with a molecular mass of 44 kDa and present in the plasma in concentrations up to 1–2 mg/mL (45 μM). AAG is generally known to have high affinity for basic drugs such as imipramine and propranolol. Other proteins that are present in lower concentration in the plasma have much lower significance in terms of the impact on the PK of the drug.

The drug–protein binding phenomenon is most commonly characterized by fraction of drug unbound (fu) (or referred to as the free fraction) in plasma or tissue and by the dissociation rate constant (K_s) that is indicative of the strength of the binding [1–4]. For a drug (D) and protein (P), the interaction is depicted below

$$[\mathrm{D}] + [\mathrm{P}] \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [\mathrm{DP}]$$

where k_1 and k_{-1} are the rate constants associated with the formation and breakdown of the DP complex. At equilibrium,

$$k_1[\mathrm{D_f}][\mathrm{P_f}] = k_{-1}[\mathrm{DP}]$$

The dissociation constant (K_s) may be defined as follows:

$$K_s = \frac{k_{-1}}{k_1} = \frac{[D_f][P_f]}{[DP]}$$

$$[DP] = \frac{[D_f]P_f}{K_s}$$

By applying law of mass action that the total drug concentration [D] is the sum of $[D_f] + [DP]$ and that the free fraction of the drug is defined as the relative concentration of free to the total concentration of the drug,

$$f_u = \frac{\text{Free drug concentration}}{\text{Total drug concentration}} = \frac{[D_f]}{[D]} = \frac{[D_f]}{[D_f] + [DP]}$$

Therefore,

$$f_u = \frac{[D_f]}{\left([D_f] + \frac{[D_f][P_f]}{K_s}\right)}$$

Dividing the numerator and denominator by $[D_f]$,

$$f_u = \frac{1}{\left(1 + \frac{[P_f]}{K_s}\right)} = \frac{K_s}{K_s + [P_f]}$$

This indicates that the fraction unbound (free fraction) is directly proportional to the dissociation rate constant. The higher the tendency of the DP complex to dissociate to release free drug, the higher will be the fraction unbound. The fraction unbound is also inversely related to the free protein concentration, in that more the concentration of free protein, the lower will be the value of fraction unbound. The unbound fraction may be expressed as values ranging from 0 to 1 or as a percentage ranging from 0% to 100%. In general, drugs with a f_u of 0.9 (90%) or more are referred to as *highly protein-bound drugs*. Although, not explicitly indicated, f_u is also dependent on the total drug concentration and in situations where the dissociation constant is small (less free drug concentration) and the plasma total drug concentrations are high, the binding sites on the protein may be saturated and f_u may increase. However, for most drugs, the f_u is essentially constant over the therapeutic drug concentration range.

Since it is only the free or unbound drug that is able to cross membranes and interact with the target tissues, distribution equilibrium is achieved when the free concentrations in both the plasma and tissue are identical. However, at this juncture, the total concentrations of drug in the plasma and tissues may be nonidentical. For example, if the fraction unbound in the plasma is 0.9 (unbound concentration of 9 units relative to a total concentration of 10 units) and that in the tissue is 0.1 (unbound concentration of 9 units relative to a total concentration of 90 units), one can appreciate that at distribution equilibrium when the free concentrations of drug are same and equal to 9 units in both the plasma and tissue, the total tissue concentration is ninefold higher. A reversal of this situation can result in plasma concentrations being ninefold higher than tissue concentrations. In general, lower the free fraction in the plasma, less is the propensity for the drug to go to tissue compartments. The primary PK parameter that

reflects this interplay is the apparent volume of distribution (V) [1,2]. The equation that states this relationship is

$$V = V_{\mathrm{p}} + V_{\mathrm{TW}} \left(\frac{f_{\mathrm{u}}}{f_{\mathrm{uT}}} \right)$$

where V_{p} and V_{TW} are the plasma and tissue water volumes, respectively, and f_{u} and f_{uT} are the fraction of drug unbound in the plasma and tissue water, respectively.

Thus, other factors being equal, drugs that have a lower free fraction in plasma tend to be sequestered in the plasma compartment (showing a low V) relative to drugs with a higher free fraction in plasma. Likewise, drugs that have a lower free fraction in tissues tend to be sequestered in the tissue compartment (showing a high V) relative to drugs with a higher free fraction in tissue.

The free drug is also the entity that is amenable to metabolism by drug-metabolizing enzymes, glomerular filtration, active secretion and active reabsorption.

The determinants of the organ metabolic clearance [1–4] are indicated by the following equation.

$$\mathrm{CL}_{\mathrm{org}} = \frac{Q_{\mathrm{b,org}} \times f_{\mathrm{u,b}} \times \mathrm{CL}_{\mathrm{int}}}{Q_{\mathrm{b,org}} + \left[f_{\mathrm{u,b}} \times \mathrm{CL}_{\mathrm{int}} \right]}$$

where $\mathrm{CL}_{\mathrm{org}}$ and $\mathrm{CL}_{\mathrm{int}}$ are organ clearance and intrinsic clearance (volume/time), Q_{org} is the organ perfusion rate or organ blood flow (volume/time) and $f_{\mathrm{u,b}}$ is fraction unbound in blood.

For drugs that are efficiently metabolized by drug-metabolizing enzymes and have high intrinsic clearance values, the above equation collapses to the following:

$$\mathrm{CL}_{\mathrm{org}} = \frac{Q_{\mathrm{b,org}} \times f_{\mathrm{u,b}} \times \mathrm{CL}_{\mathrm{int}}}{f_{\mathrm{u,b}} \times \mathrm{CL}_{\mathrm{int}}}$$

$$\mathrm{CL}_{\mathrm{org}} = Q_{\mathrm{b,org}}$$

The organ clearance is thus perfusion rate limited and independent of both protein binding and intrinsic clearance.

For drugs that are not good substrates for drug-metabolizing enzymes (low intrinsic clearance), the equation for organ metabolic clearance then rearranges to the following:

$$\mathrm{CL}_{\mathrm{org}} = \frac{Q_{\mathrm{b,org}} \times f_{\mathrm{u,b}} \times CL_{\mathrm{int}}}{Q_{\mathrm{b,org}}}$$

$$\mathrm{CL}_{\mathrm{org}} = f_{\mathrm{u,b}} \times \mathrm{CL}_{\mathrm{int}}$$

The free fraction in blood or $f_{\mathrm{u,b}}$ is now a crucial determinant of hepatic metabolic clearance in addition to the low intrinsic capacity (intrinsic clearance) of drug-metabolizing enzymes that can biotransform the drug.

As an extension, organ clearance can also be expressed as

$$\mathrm{CL}_{\mathrm{org}} = Q_{\mathrm{b,org}} \times \mathrm{ER}$$

where ER is the extraction ratio and is the fraction of drug that is removed by the eliminating organ per single pass of the blood through the organ [1,2,4]. As a corollary,

the fraction that escapes the elimination organ is given by [1 - ER]. This relationship allows one to estimate the expected bioavailability (F) of an orally administered drug.

$$F = f_a \times f_g \times f_h$$

where f_a, f_g, and f_h are fractions that escape the lumen, gut wall metabolism, and hepatic metabolism, respectively. If we assume that all of drug escapes the lumen and gut wall metabolism, f_h will then determine the lower limit of the oral bioavailability. Thus,

$$F = 1 \times 1 \times f_h = f_h$$

But,

$$F = f_h = 1 - ER$$

$$F = 1 - \left[\frac{f_{u,b} \times CL_{int}}{Q_{b,org} + \left(f_{u,b} \times CL_{int}\right)} \right]$$

$$F = \frac{Q_{b,org}}{\left[Q_{b,org} + \left(f_{u,b} \times CL_{int}\right)\right]}$$

For a low clearance drug (low CL_{int}), this equation collapses to

$$F = \frac{Q_{b,org}}{Q_{b,org}} = 1$$

For a high clearance drug (high CL_{int}), this equation collapses to

$$F = \frac{Q_{b,org}}{\left(f_{u,b} \times CL_{int}\right)}$$

Thus, a higher fraction unbound can lower the overall bioavailability of the drug. Overall, f_u can significantly impact hepatic clearance, oral bioavailability, and total body clearance (CL).

Similarly, the overall filtration clearance of a drug in the kidney is given by $f_u \cdot$ GFR (glomerular filtration rate) [1,2]. Furthermore, active secretion and active reabsorption that are mediated by specific efflux or uptake transporters are also able to accept only unbound drug for transport, and thus, the unbound fraction of drug impacts these processes as well.

To summarize, polytherapy or disease states that alter the drug–plasma and/or drug–tissue binding due to changes in the concentration of the plasma/tissue-binding proteins or displacement of bound drug from its binding sites can result in changes in the plasma free concentrations of the drug and potentially lead to changes in primary PK parameters (V and CL) and consequently the derived PK parameters, such as half-life and AUC, of a victim drug.

During the discovery and development of an NCE (new chemical entity), determination of the plasma protein binding or HSA binding of a NCE is thus crucial to

aid the complete PK profiling of the drug and evaluation of the drug–drug interaction potential of the NCE.

12.2 ESTIMATION OF PROTEIN BINDING

Several *in vitro* methods are used for the estimation of the unbound drug fraction (f_u) [5,6] and include equilibrium dialysis, ultrafiltration, ultracentrifugation, and so on (Fig. 12.1). Among them, equilibrium dialysis and ultrafiltration are most commonly used for the estimation of f_u in plasma, serum, or diluted tissue homogenate because of their simplicity and high throughput compatibility. However, neither of these methods is free of biological, chemical, or physical artifacts, and the advantages and disadvantages of each method are discussed in this section.

Often, in drug discovery and development, the choice of method is based on progress stage of the compound. As the compound progresses in its life cycle, the methods become more robust and reliable (Fig. 12.2). In early drug discovery, rapid screening methods are required, which allow classification of compounds in low, medium, and high binding categories. As the compound moves forward and more quantity is available, more definitive methods such as equilibrium dialysis and ultrafiltration are used, which allow the study of sex or species differences in plasma protein binding, linearity in binding with respect to drug and protein concentration and identification of proteins to which drugs bind. It is recommended that protein binding should be studied by at least two methods for compounds in this stage and the binding estimations are supported by sensitive and specific analytical methods [e.g., liquid chromatography tandem mass spectrometry (LC-MS/MS)]. For compounds in clinical development,

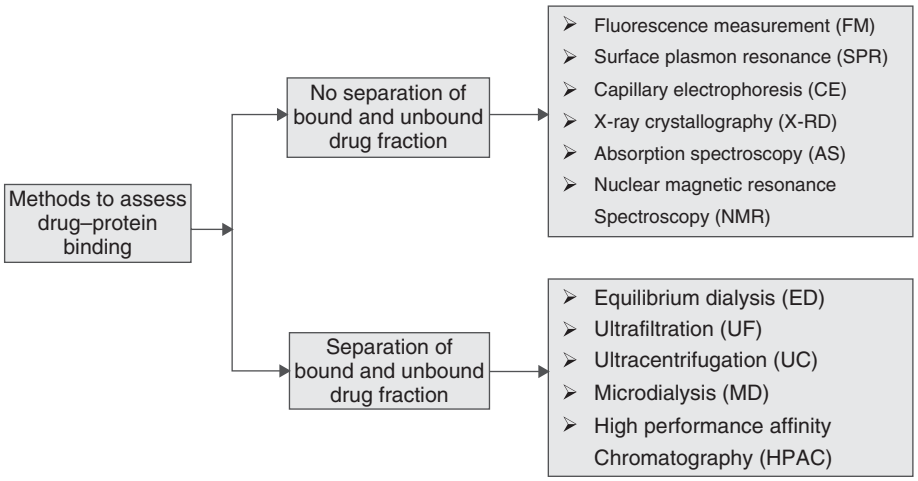

Figure 12.1 Methods used to assess protein binding of drugs. Methods for estimation of plasma protein binding involve either prior separation of bound and unbound drug [bottom right quadrant of the figure] or determination without prior separation of bound drug from free drug [top right quadrant of the figure]. Routine methods use the separation of free and protein-bound drugs for estimation of the fraction unbound.

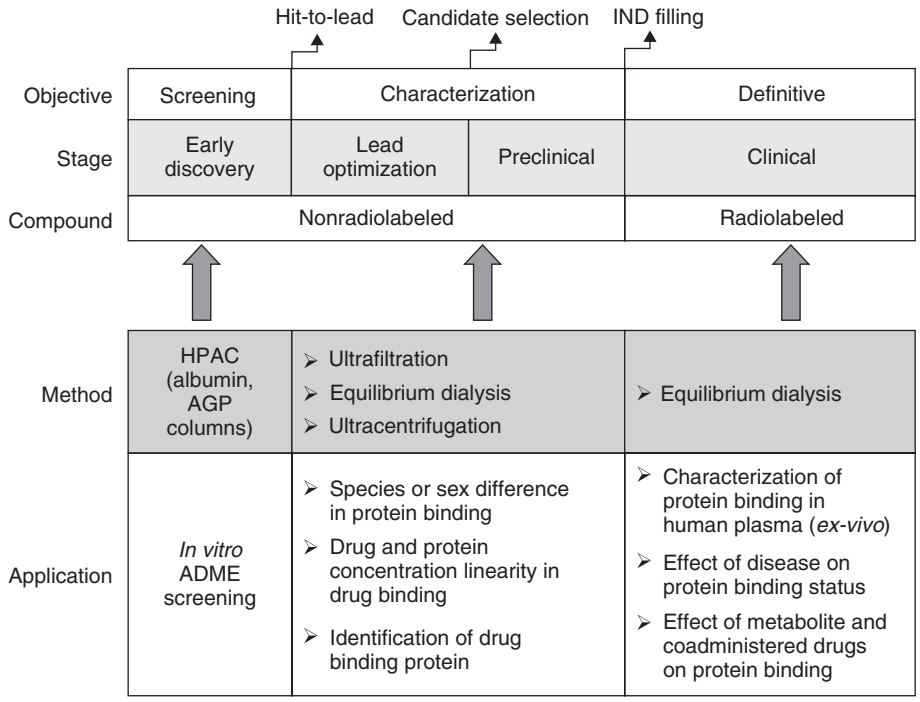

Figure 12.2 Protein binding methods and their application in various stages of the drug development. The method used to assess protein binding is dependent on the depth of information required. At early stages, quick and semiquantitative estimation of the fraction unbound is sufficient, but during the clinical development phase, an accurate estimate of protein binding is done using equilibrium dialysis—"the gold standard" as it also has the potential for evaluation of protein-binding-based drug–drug interaction.

radiolabeled compounds that provide a more reliable and robust assessment of protein binding are used.

12.2.1 Equilibrium Dialysis

Equilibrium dialysis method is often considered as the "gold standard" for measuring protein binding. It is based on the establishment of an equilibrium state between a protein compartment and a buffer compartment, separated by a semipermeable membrane. This membrane (pore size/molecular weight cutoff (MWCO) is selected depending on the compound to be studied or application *viz.* 12–14K MWCO, 6–8K MWCO, 10K MWCO, 3.5K MWCO, etc.) and allows only low molecular weight ligands such as drug molecules to be transported between two compartments. The experimental method is depicted in Fig. 12.3.

Briefly, drug-spiked biological matrix (plasma, serum, or tissue homogenate) is placed in one compartment and buffer (typically sodium or potassium phosphate buffer, pH 7.4) is placed in the other, and the assembly is incubated at 37 °C until equilibrium between free drug concentration in matrix and that in the buffer is reached (incubation period usually lasts longer than 2 h). During the incubation period, in addition to the

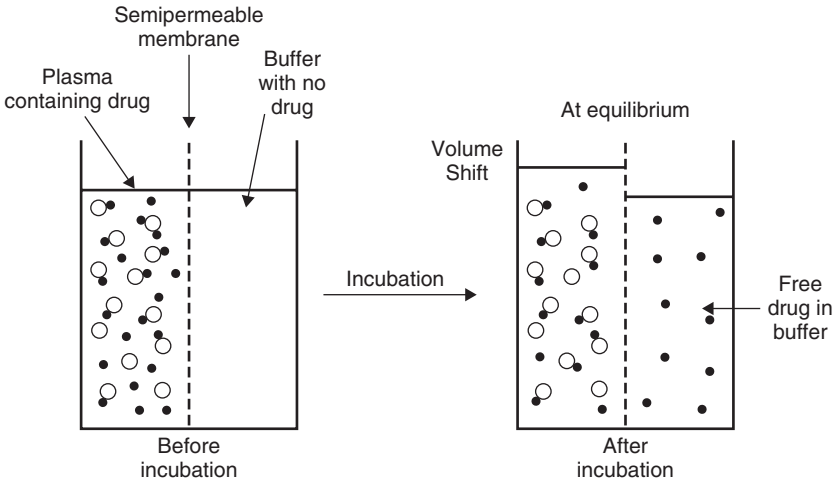

Figure 12.3 Equilibrium dialysis method for estimating protein binding of drugs. The figure indicates the movement of free drug (closed circle) from the left reservoir that contains both free drug and drug bound to protein (open circle), through the semipermeable membrane, to the right reservoir that contains only buffer. At equilibrium, the free drug concentrations on both sides are the same. Note the volume shift due to movement of buffer to the left side protein-containing chamber due to osmotic pressure and/or Donnan ion effect.

transport of unbound drug molecules, water molecules from the buffer compartment are continuously moving to the matrix compartment because of the differences in osmotic pressure between the two compartments and/or the Donnan ion effect. This results in an increase in volume in the matrix compartment and decrease in volume in the buffer compartment; this phenomenon is known as the *volume shift*, which is usually about 15–20% and needs to be corrected during the estimation of protein binding. At equilibrium, aliquots of matrix and buffer are sampled for analysis of drug concentrations. Also volumes in the matrix and buffer compartments are measured. Protein binding is estimated as shown in Equation 12.1.

$$\text{Protein binding } (\%) = \frac{\left(C_{\text{pe}} - C_{\text{be}}\right) \times \frac{V_{\text{pe}}}{V_{\text{pi}}}}{\left[\left(C_{\text{pe}} - C_{\text{be}}\right) \times \frac{V_{\text{pe}}}{V_{\text{pi}}}\right] + C_{\text{be}}} \tag{12.1}$$

where C_{pe} is the peak area of analyte in matrix at equilibrium, C_{be} the peak area of analyte in buffer at equilibrium, V_{pe} the volume of matrix at equilibrium, and V_{pi} the initial volume of matrix.

Maintenance of physiological pH and temperature is important for accurate estimation of protein binding [7,8]. High throughput equilibrium dialysis apparatus have become increasingly popular and are widely accepted today. These include the Rapid Equilibrium Device (RED, Thermo Scientific) [9], Microequilibrium Dialysis Device HTD 96 (HTDialysis) [10], 96-Well Equilibrium Dialyzer (Harvard Apparatus) [11], and so on. These require shorter preparation and dialysis times and are amenable to automation. For bioanalysis, after attainment of equilibrium, an aliquot of buffer

(90 μL) from the buffer compartment is mixed with blank matrix (10 μL) and similarly, an aliquot of matrix (10 μL) from the matrix compartment is mixed with blank buffer (90 μL) before sample processing. This approach eliminates the need for preparation of separate calibration curves of drug in buffer and matrix and can save valuable analytical instrumentation time. For calculation of protein binding, the values are corrected for sample dilution by applying respective dilution factors, that is, values from matrix side are multiplied by 10 and values from the buffer side are multiplied by 1.1. The fraction of unbound drug (f_u) is calculated as shown in Equation 12.2.

$$f_u = \frac{C_u}{C_b} \tag{12.2}$$

where C_u is the concentration of drug on buffer side and C_b the concentration of drug on matrix side.

The percentages of drug unbound or free and bound to protein are calculated using Equations 12.3 and 12.4.

$$\% \text{ Drug Unbound} = f_u \cdot 100 \tag{12.3}$$

$$\% \text{ Protein binding} = (1 - f_u)100 \tag{12.4}$$

The correction for volume shift is not generally used in the 96-well setup because of the large error associated with withdrawal and measurement of small volumes required for the correction. When nonradiolabeled drug is used, drug concentrations in matrix and buffer are typically analyzed by LC-MS/MS, and when a radiolabeled drug is used, analysis is performed by liquid scintillation counting (LSC).

Equilibrium dialysis can also be used to study the binding interactions by dialyzing free drug through a semipermeable membrane until its concentration across the membrane is at equilibrium (Harvard Apparatus). Data obtained under different experimental conditions can then provide information on various binding parameters such as binding constants and number of binding sites or binding capacity. Binding to HSA and AAG can be studied by this method using purified protein solutions [12].

Equilibrium dialysis is theoretically the most accurate method for determining plasma protein binding because the equilibrium is not shifted when aliquots are taken from either side of the membrane [13]. It is a suitable method for drugs that are highly protein bound (>98%). Some advantages and disadvantages of equilibrium dialysis method are listed in the following.

Advantages

- Easy to setup and use;
- Needs small amount of sample;
- Temperature controlled;
- Thermodynamically sound;
- Reproducible results can be obtained even for low affinity compounds;
- Meaningful results can be determined for compounds with nonspecific binding (NSB) [14];
- Compatible to high throughput with robotic systems;
- Binding interactions can be studied.

Disadvantages

- Requires long time to reach equilibrium;
- Volume shift [15];
- Donnan effects (hindering of the passage of free ligand) [16];
- NSB;
- Potential degradation of compound in matrix due to thermal or metabolic instability;
- Shift of pH due to continuous loss of carbon dioxide [17];
- Overestimation of free fraction as a result of slight leakage of protein into buffer compartment [18].

12.2.2 Ultrafiltration

Ultrafiltration method is based on the separation of unbound drug from the protein-bound drug by filtering the protein–drug matrix through a semipermeable membrane under positive pressure generated by centrifugation. The experimental method is depicted in Fig. 12.4.

Briefly, drug-spiked matrix is placed in the sample reservoir, and the reservoir is capped and fitted onto a sample collection tube. The entire ultrafiltration assembly is then centrifuged at $1000-2000\times g$. The incubation time is fixed based on the flow rate such that the ultrafiltrate (plasma water, PW) obtained is about one-fifth of the initial volume of the sample. The centrifugation can be carried out at room temperature or at 37°C; however, the chosen temperature should be maintained constant during the centrifugation process as drug binding decreases with rising temperature. A major

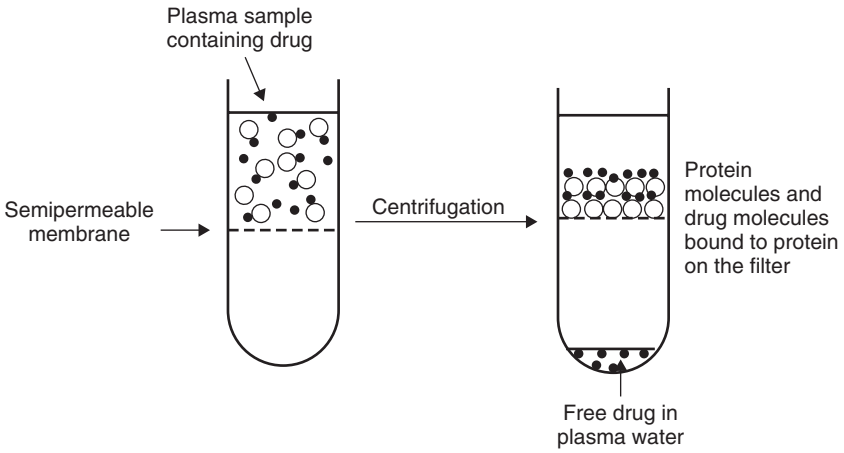

Figure 12.4 Ultrafiltration method for estimating protein binding of drugs. The figure indicates the movement of free drug (closed circle) from the top chamber that contains both free drug and drug bound to protein (open circle), through the ultrafiltration membrane, to the bottom chamber. The movement is assisted by the application of a centrifugal force. The ultrafiltrate is analyzed to obtain the free drug concentration.

drawback of the ultrafiltration method is the potential NSB of the drug to the plastic tube and ultrafiltration membrane, which will lead to an underestimation of the free drug concentration. This is usually assessed and corrected when calculating the protein binding by running a parallel control sample using drug-spiked blank PW or physiological phosphate-buffered saline and using this as sample instead of plasma. The concentration of the drug in the ultrafiltrate and plasma can be quantified using a LC-MS/MS. If radiolabeled drug is used, LSC can be used. The fraction of drug unbound can be calculated by using Equation 12.5 (employs correction factor for NSB, that is, ratio between drug concentration in the original PW (C_{pw}) and the concentration of the drug in PW ($C_{pw,f}$) after ultrafiltration).

$$f_u = \frac{C_f \left(C_{pw}/C_{pw,f}\right)}{C_p} \tag{12.5}$$

where C_f is the concentration of drug measured in the ultrafiltrate after centrifugation of the matrix containing the drug and C_p is the concentration of the drug in the matrix before centrifugation.

The percentage of drug bound or unbound can be calculated by using Equations 12.3 and 12.4 described earlier.

To reduce NSB of drugs, pretreatment of the filter membrane with 5% Tween 80 or benzalkonium chloride has been suggested [19]. Equilibrium dialysis is considered to be more suitable for protein binding estimations if the NSB of the drug to the ultrafiltration apparatus is greater than 5%. It has also been suggested that if NSB exceeds 5%, correction for NSB for estimating the protein binding of drugs should not be used, as the presence of plasma may alter its extent.

Ultrafiltration can be performed in a single ultrafilter unit or in a 96-well plate high throughput ultrafiltration system: Ultrafiltration unit (Millipore).

Some advantages and disadvantages of ultrafiltration method are listed in the following.

Advantages

- Fast, simple, and efficient technique;
- Lack of sample dilution and volume shifts;
- No requirement of nonphysiological buffer;
- Compatible to high throughput with robotic systems.

Disadvantages

- NSB of drug to plastic tube or ultrafiltration membrane;
- Constriction of membrane pores during ultrafiltration;
- Leakage of bound drug through the membrane;
- Donnan ion effect;
- Volume of ultrafiltrate may not be sufficient for assay;
- Deviation of drug–protein binding equilibrium because of the change in protein concentration due to loss of plasma water; especially in case of high protein-bound drugs.

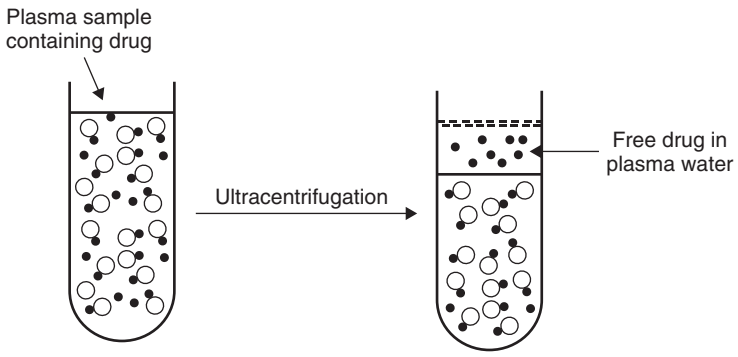

Figure 12.5 Ultracentrifugation method for estimating protein binding of drugs. The figure indicates the sedimentation of drug bound to protein (open circle) from the free drug (closed circle) under the influence of an extremely high centrifugal force ($150,000 \times g$) and a positive air pressure of up to 30 psi. Sampling of supernatant yields the free drug concentration.

12.2.3 Ultracentrifugation

Ultracentrifugation is based on the separation of free and protein-bound drug by application of a high centrifugal force without a membrane. The method is based on differential sedimentation of plasma constituents depending on their molecular mass. The experimental method is depicted in Fig. 12.5.

Briefly, drug is spiked in plasma and aliquots are transferred to centrifuge tubes for centrifugation at $150{,}000 \times g$ at an air pressure of 30 psi. The remaining plasma is maintained during the time of centrifugation. The centrifugation time is predetermined by stopping the centrifuge at different time intervals and measuring the drug concentration in the top layer. The time at which the concentration is constant is chosen for the centrifugation of the study samples. At the end of the centrifugation period, the plasma separates into distinct layers: the sedimented plasma protein layer, the supernatant PW layer, and a thin white lipoprotein layer at the surface. The drug concentrations are estimated in the supernatant PW layer ($C_{\text{supernatant}}$) and the stored spiked plasma (C_{initial}) by LC-MS/MS analysis. The fraction of drug unbound can be calculated by using Equation 12.6. The percentage of drug bound or unbound can be calculated by using Equations 12.3 and 12.4 described earlier.

$$f_{\text{u}} = \frac{C_{\text{supernatant}}}{C_{\text{initial}}} \tag{12.6}$$

Depending on the centrifuge available, different centrifugation times have been used. Also a microscale ultracentrifugation was evaluated by Nakai *et al*. [20], which utilized only 200 μL of plasma sample with a bench top ultracentrifuge ($436,000 \times g$, 140 min). They showed a very good correlation in the protein binding estimated for 10 compounds with this method and that determined by equilibrium dialysis or ultrafiltration. Ultracentrifugation is an alternative method to equilibrium dialysis and ultrafiltration and can be used for compounds having high NSB, as it eliminates problems associated with free membrane effects. Also, it is the best method for highly hydrophobic compounds for which other methods prove inadequate. Some advantages and disadvantages of ultracentrifugation method are listed in the following.

Advantages

- Fewer NSB issues;
- No dilution of sample.

Disadvantages

- Expensive instrumentation (high speed ultracentrifuge);
- Time consuming (up to 12–24 h centrifugation time);
- Low throughput as limited samples can be centrifuged simultaneously;
- No automation possible;
- Larger volume of sample required;
- Self-sedimentation of large-molecular-weight compounds;
- Interferences due to thermal back diffusion and floating lipoprotein in the supernatant.

12.2.4 High Performance Affinity Chromatography

High performance affinity chromatography (HPAC) for estimation of protein binding of drugs is based on the use of a liquid chromatographic technique that uses a protein as the stationary phase for the analysis of sample containing drug. The retention of the drug is based on its reversible interaction with the protein (affinity ligand). The earliest work using HSA columns was carried out by Domenici *et al*. [21]. Later, different strategies for the development of columns immobilized with HSA have been reported. The epoxy method used the epoxy groups of a copolymer (glycidyl methacrylate and ethylene dimethacrylate) for direct immobilization of HSA through its amine residues. In other approaches, the epoxy groups were converted to diols for later use in the carbonyldiimidazole, disuccinimidyl carbonate, and Schiff base methods [22]. Stable and selective chiral stationary phases were also prepared by covalent binding of HSA to silica particles via reactive polymers. Stationary phases obtained by immobilization of HSA on (C8) and (C18) reverse phases and on poly(1-vinylimidazole)-coated silica have also been reported [23]. In addition to HSA, other columns using immobilized proteins such as AAG and lipoproteins of different species are now available.

HPAC can be used to study protein binding of drugs using zonal elution and frontal analysis techniques. In both these methods, the protein of interest is used as an immobilized ligand and an injection or application of analyte is made onto the affinity column in the presence of only buffer or buffer plus a mobile phase modifier/competing agent. By examining the elution time or volume of the analyte after it has passed through the column, it is possible to obtain information on the extent of drug–protein binding, the equilibrium constants for these processes, the ability of the drug to compete with other compounds, the effects of temperature or solvent composition on these reactions, and the structure and location of the drug binding sites on the immobilized protein [24].

Some advantages and disadvantages of HPAC method are listed in the following.

Advantages

- Easy setup;
- Low cost;
- Low sample consumption.

Disadvantages

- Only binding to one protein at a time is possible that may lead to underestimation of protein binding of drug;
- For UV-inactive compounds, LC-MS/MS is needed.

12.2.5 Microdialysis

Microdialysis is a technique for *in vivo* sampling of drugs that has been used to estimate the concentrations of unbound drug present in the extracellular fluids in various tissues or organs or in the blood. The data obtained from microdialysis sampling is continuous unlike that obtained from blood sampling at specific time points, and thus, it is best represented on a time axis at the midpoint of the sampling interval [25]. A schematic describing the functioning of a microdialysis probe is shown in Fig. 12.6.

The basic setup for a microdialysis experiment consists of a microdialysis probe, a perfusion pump, and an analytical method with the required sensitivity to quantify small concentrations of substances in small volumes of sample. Briefly, a microdialysis probe, consisting of a capillary (typical outer diameter of 500 μm) with a dialysis membrane covering its tip, is implanted into a blood vessel, tissue, or organ. The probe is then perfused with a physiological buffer such as Ringer's solution at a constant flow rate

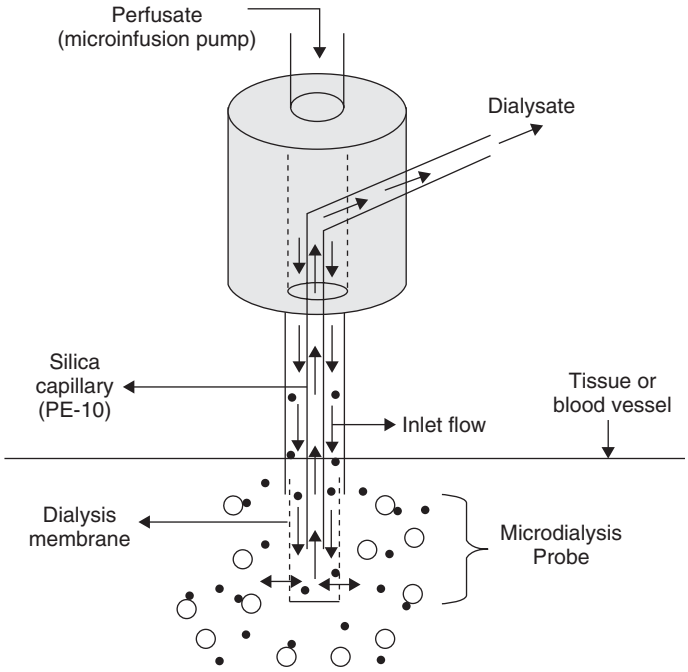

Figure 12.6 Microdialysis probe design and functioning. The figure indicates the movement of free drug from the tissue/blood vessel through the tip of the microdialysis probe that has semipermeable membrane. Using this method, after drug administration, the change in concentration of free drug can be monitored continuously over time akin to blood sampling during traditional pharmacokinetic studies.

(usually $<2\,\mu$L/min). Owing to the semipermeable membrane of microdialysis with a typical MWCO of 20 kDa, only small molecules diffuse along the concentration gradient toward the probe lumen into the perfusate. The concentration of the drug in the perfusate is analyzed by LC-MS/MS and represents the unbound drug concentration.

When using microdialysis, it is important to assess the recovery of drug. Microdialysis is not performed under equilibrium conditions because the perfusate is constantly being pumped through the probe; thus, the concentration of the drug in the perfusate is always lower than that of the extracellular fluid surrounding the dialysis probe. The ratio between these concentrations is defined as relative recovery of the analyte. The relative recovery decreases as the flow of the perfusate increases. To improve recovery of drugs, bovine serum albumin, glycerol [26], or β-cyclodextrin [27] have been added in the perfusate by some researchers.

Microdialysis allows continuous sampling from awake, freely moving animals in small numbers. Moreover, multiple site sampling can provide detailed PK information and drug transport equilibration across membranes such as blood-to-brain distributions. The microdialysis technique is devoid of any loss of biological fluid, thus enables use of the same animals as their own control in a cross-over design.

Some advantages and disadvantages of microdialysis method are listed in the following.

Advantages

- Can be used to estimate unbound drug concentrations (and kinetics) in blood or tissue in awake animals thus maintaining physiological conditions;
- Very clean dialysate requiring no sample clean-up;
- Unbound drug in the dialysate is chemically stable as the dialysate is free of any plasma or tissue components.

Disadvantages

- Low recovery of hydrophobic and highly protein-bound drugs;
- *In vitro* recovery may not translate to *in vivo* recovery due to interaction of plasma or tissue components with membrane material;
- Requirement of sensitive analytical methods that can work with low sample volumes;
- Tissue damage by microdialysis probe implantation.

12.2.6 Other Methods

Many other nonseparative methods have been used to assess protein binding of drugs. These include fluorescence measurement, surface plasmon resonance, capillary electrophoresis (CE), X-ray crystallography, absorption spectroscopy, and nuclear magnetic resonance spectroscopy.

The fluorescence measurement technique [28] uses the detection of intrinsic fluorescence of tryptophan (Trp) residues of HSA and AAG, which are the primary plasma proteins. In tryptophan fluorescence quenching (TFQ), the Trp is excited at 285 nm and the emission fluorescence is monitored as the drug is titrated into the system. The fluorescence of the intrinsic Trp in HSA is quenched by the drugs that

interact closely to the Trp. The binding constant is measured by fitting the drug concentration versus fluorescence profile. TFQ is a domain-specific binding assay that is susceptible to interference from fluorescent compounds. It cannot measure the binding constants for compounds that bind at sites distant from the Trp such that quenching is not detected. The dissociation constant K_D of analyte can then be calculated. This method can be used as a 96-well assay and is high throughput compatible.

Another high throughput method uses surface plasmon resonance biosensors [29], such as Biacore®, to study drug–plasma protein binding. In this method, one interacting partner ("ligand") is attached to the surface of a chip and the sample containing the second interaction partner ("analyte") is passed over the surface of the chip. Binding of molecules to the sensor surface generates changes in the resonance angle in real time, which is proportional to the bound mass. Drugs can be classified as low, medium, or high binding, and this process can be applied to large numbers and classes of compounds. When additional rigor is desired and sufficient compound is available, definitive K_D constants can be determined. The limitations of Biacore® are nonaqueous solvent effects and lack of ruggedness.

Drug–protein binding can also be studied using CE. Advantages of CE methods over chromatographic methods include its resolving power and small amounts of proteins and drugs required. The major limitation of the CE methods is their low sensitivity. In the electrophoretic techniques, the separation of the free drug, the free protein, and the drug–protein complex is based on their differences in electrophoretic mobility due to the net charge and size differences. It is simple, robust, and easy to implement; can deal with multiple equilibria; and requires less reagents than all the other methods [30].

12.3 SUMMARY

The phenomenon of plasma and tissue binding is the important determinant of the overall properties of the drug that are of importance in evaluating the PK and PD of drugs and the design of the dosage regimen. There are several methods for the estimation of plasma protein binding, and one must carefully choose the method that is commensurate with the level of information regarding plasma protein binding that is desired based on the developmental stage of the NCE or drug.

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