23 Plasma Protein Binding Methods in Drug Discovery and Development: Bioanalysis

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23.1 SUMMARY

This chapter provides an overview of both conventional and unconventional methods to determine plasma protein binding. Plasma protein binding is an important ADME parameter required to understand drug efficacy and possibly toxicity. Plasma protein binding refers to the noncovalent interaction of drugs with plasma proteins such as albumin and α-acid glycoprotein (α-AGP). It may contribute to a wide variety of ADME phenomena such as drug–drug interaction potential and nonlinear, stereoselective, or interindividual pharmacokinetics variability. Therefore, the potential exists to identify and differentiate drug candidates based on plasma protein-binding values. Experiments using plasma protein binding advance our understanding of ADME properties to aid in candidate selection and development by determination of the unbound drug blood concentrations as well as (potentially) drug concentration at the site of action.

Recent progress in automation as well as increased routine performance of the analytical instrumentation has enabled a greater number of compounds to be assayed earlier in discovery, often at a high enough quality level such that repeat determinations later in development are not needed. Significant effort has been invested to search for high throughput screening methods that reliably and accurately determine protein binding in early drug discovery. The development of 96-well formats for equilibrium dialysis, ultrafiltration, and ultracentrifugation, combined with liquid handling automation have

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transformed a formerly tedious, labor-intensive assay into a process, which can now be swiftly conducted. In addition, new approaches, referred to as *emerging techniques*, are also under investigation, including chromatographic methods such as the human serum albumin (HSA) column and spectroscopic methods such as surface plasma resonance, solid-phase microextraction, Transil membrane screening, and capillary electrophoresis (CE) methods.

Significant challenges still remain unsolved, particularly in cases of high protein binding with low exposure levels that fall below the normal limit of quantitation (LOQ). This chapter describes the impact of high (>99%), undeterminable protein binding on compound developability as well as possible solutions including plasma dilution techniques.

23.2 INTRODUCTION

The use of *in vitro* ADME (absorption, distribution, metabolism, and excretion) tools offers the exciting prospect of optimizing a drug candidate's efficacy while potentially reducing costly attrition during drug development. Over the last two decades, enormous investment and interest have blossomed in high throughput *in vitro* ADME screens, such as permeability, metabolic stability, drug–drug interaction potential, and transporter affinity. From a hierarchical perspective, *in vitro* methods of plasma protein binding for drug candidates have been less preferred and less frequently performed than other ADME screens. However, as the value of plasma-protein-binding data has become better understood, and, as more tools have been developed to conduct the experiment in a higher throughput manner, the conduct of plasma-protein-binding assays has increased in the early discovery or lead optimization space.

Plasma protein binding refers to the noncovalent interaction of drugs with plasma proteins such as albumin and α_1-acid glycoprotein (α-AGP). The premise that only unbound or free drug is available to exert a pharmacological effect is known as the *free drug hypothesis* [1,2]. Although there is controversy in the literature, unbound drug levels are generally regarded to be the principal determinant of tissue distribution, cell entry, receptor interactions, and availability for elimination, thus impacting the pharmacokinetic/pharmacodynamic (PK/PD) relationship or off-target adverse reactions or toxicities. Plasma protein binding may contribute to a wide variety of ADME phenomena such as drug–drug interaction potential and nonlinear, stereoselective, or interindividual pharmacokinetics variability. Therefore, the potential exists to identify and differentiate drug candidates based on plasma-protein-binding values [3,4]. Plasma-protein-binding experiments advance our understanding of ADME properties to aid in candidate selection and development by determination of the unbound drug blood concentrations as well as (potentially) drug concentration at the site of action.

In 2002, the clinical relevance of plasma protein binding was challenged by Benet and Hoener [5], who through careful study demonstrated that protein binding is relevant primarily for IV administered drugs with a high extraction ratio and oral drugs with a high extraction ratio and a nonhepatic clearance mechanism. Examination of data for 456 currently marketed drugs showed that protein binding influenced exposure for 25, which fell into the two categories described above. If the therapeutic index is considered, protein binding influences even fewer compounds. Benet and Hoener's work might lead some to conclude that the protein-binding experiments are not worth

the time and effort they consume. However, the clear need for protein binding in the discovery and preclinical development stages to conduct allometric scaling and understand species-difference behavior is well understood. In rare cases, differences between animal and human plasma-protein-binding values may lead to significant differences in observed clearance, with severe negative outcomes. Protein-binding behavior of a drug candidate may not be in and of itself decision making, but has a profound influence on a variety of *in vivo* and *in vitro* properties during ADME and pharmacology experiments. Thus, its determination early in drug discovery can provide significant input into understanding tissue distribution and pharmacological effect.

Before the last decade, a significant stumbling block of plasma-protein-binding methods has been lack of miniaturization and automation. Plasma protein binding has been traditionally performed by equilibrium dialysis during drug development using ^{3}H- or ^{14}C-labeled compounds. The timing of these experiments is largely determined by the effort and expense to obtain radiolabeled material. The traditional methods consume a significant amount of material, are very labor intensive and not easily automated, but are considered sufficiently rigorous to permit data inclusion in regulatory submissions.

Significant effort has been invested to search for high throughput screening methods that reliably and accurately determine protein binding in early drug discovery. The development of 96-well formats for equilibrium dialysis and the coupling of this with liquid handling automation have transformed a formerly tedious, labor-intensive assay into a process that can now be swiftly conducted. However, as 96-well techniques are validated, the need for the final low throughput "definitive" radiometric experiment is becoming less and less compelling. In some situations, experimental results derived

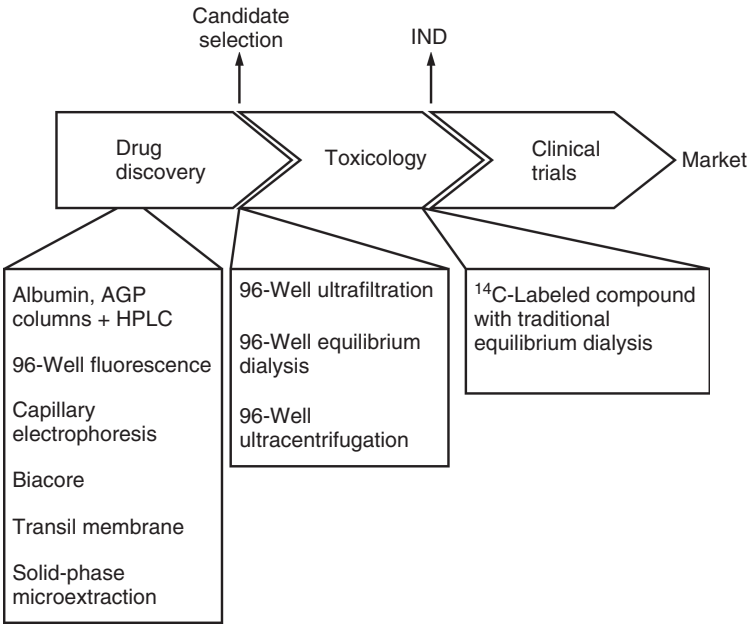

Figure 23.1 Overview of plasma-protein-binding methods during drug discovery and development.

from LC/MS/MS analysis may be used in regulatory submissions. In addition, new approaches referred to as *emerging techniques* are also under investigation and they will be described later.

An overview of normal timings of the techniques, which will be discussed are shown in Fig. 23.1. Early in drug discovery, rapid screening techniques that allow binning into low, medium, and high binding categories may be utilized. As a potential drug candidate progresses forward in the drug discovery process, and more of it is synthesized, more definitive approaches such as equilibrium dialysis are applied. Once a drug candidate is deemed to have a high likelihood of success, traditional equilibrium dialysis using radiometric detection may be performed. However, the approaches used across the pharmaceutical industry continue to evolve. With significant recent improvements in the high throughput automated devices used to conduct the incubations as well as the analytical instrumentation, the need for the "definitive" radiometric experiment is declining. Some companies may also choose not to invest resources in the conduct of binning experiments if higher quality data can be obtained with appropriate turnaround time from the 96-well equilibrium dialysis, ultrafiltration, or ultracentrifugation experiments. The maturation of plasma-protein-binding assays continues to be both an interesting and dynamic process.

23.3 TRADITIONAL METHODS

Plasma protein binding has been conducted for several decades as part of normal compound progression from animal experiments into the clinic. The original manually conducted experiments relied on simple approaches utilizing common laboratory equipments such as the centrifuge. An overview of the experimental process for "traditional" techniques [6], based on their popularity and longevity of conduct, that is,

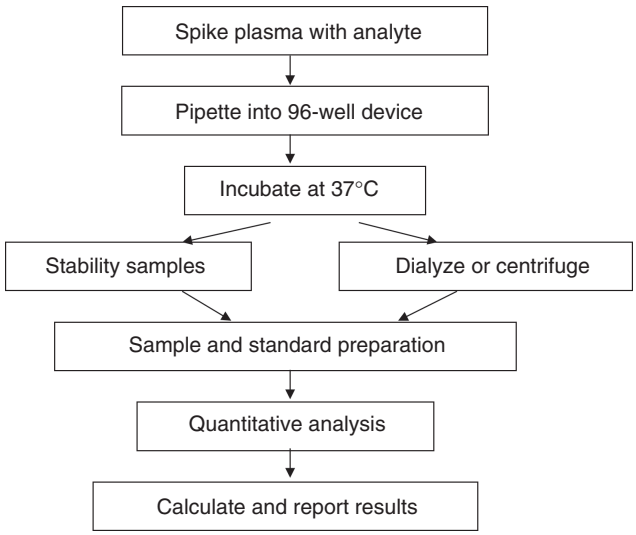

Figure 23.2 Process flow for traditional methods of plasma protein binding: ultrafiltration, ultracentrifugation, and equilibrium dialysis.

ultrafiltration, equilibrium dialysis, and ultracentrifugation is shown in Fig. 23.2. In addition, a summary of the advantages and issues, as well as, comparison with more innovative or higher throughput approaches described later in this chapter, is shown in Table 23.1.

When embarking on a protein-binding assay, several variables should be considered in the experimental design. The choice of which preclinical species to examine should be dictated by the purpose of the protein-binding experiments, such as PK calculations, PK/PD relationship, or exposure margin calculations for safety assessment studies. Plasma protein binding should be evaluated over an anticipated therapeutically and/or toxicologically relevant concentration range for the compound of interest. Additionally, these methods can be used to investigate individual binding proteins (i.e., HSA, α-AGP, preclinical species' albumin). Plasma harvested using EDTA as an anticoagulant is recommended. Concerns have been raised that the anticoagulant heparin may interfere with protein binding, and it should thus be avoided. During preparation of initial analyte-spiked plasma, minimal organic content (<2% by volume) should be maintained to minimize interference with protein binding. The stability of the compound in all species and matrices, including buffer solutions, should be assessed over the time period used for equilibration. This is accomplished by incubating an aliquot of the matrix standard and spiked buffer at the lowest concentration under the same conditions as the equilibrium dialysis apparatus. Samples taken at time zero and at the end of experiment should be assessed to determine percentage of analyte remaining.

23.3.1 Ultrafiltration

Ultrafiltration experiments [7–10] have been conducted in 96-well format for over a decade, thanks to the commercial availability of the filtration plates. In this experiment, an aliquot of analyte-spiked plasma is pipetted into an individual well of a single-use 96-well device. The bottom of this well is composed of a filtration membrane with a 10,000 Da molecular weight cutoff. The entire device is then centrifuged, during which time the unbound analyte solvated in plasma water and other low molecular weight components, will migrate through the filter to a collection well on the opposite site of the filter. Bound analyte will remain with the plasma proteins on the top half of the device, which is then discarded. The analyte-containing filtrate samples are then quantitatively assayed, in addition to unfiltered plasma spiked with analyte, once the centrifugation cycle in finished. The analyte percentage bound is then calculated using the following formula:

$$\left[\%\ \text{Unbound} = \frac{\text{Drug concentration in ultrafiltrate post centrifugation}}{\text{Drug concentration in plasma pre centrifugation}} \times 100\right]$$

Ultrafiltration is a relatively quick, inexpensive, and simple experiment to conduct but does suffer serious problems and inaccurate values due to compound adsorption to the top half of the filtration device. This can lead to serious overestimation of protein binding.

23.3.2 Equilibrium Dialysis

Equilibrium dialysis is generally regarded as the gold standard method for obtaining plasma-protein-binding information [11–16]. In addition to improvements in

TABLE 23.1 Comparison of *In Vitro* Methods of Plasma Protein Binding

Techniques	Advantages	Issues
Traditional Techniques		
96-Well ultrafiltration	% Binding Commercially available plates Automated	Nonspecific binding Must correct for volume shifts Not true equilibrium experiment
96-Well equilibrium dialysis	% Binding Automated "Gold standard" Flexible	May require long equilibration times Long-term plasma stability of analyte at 37°C may be problematic Devices not widely available
96-Well ultracentrifugation	% Binding Automated	Requires ultracentrifuge or long centrifugation times May generate errors because of lipoprotein interactions in supernatant
Screening Techniques		
Chromatographic methods (Human Serum Albumin, α-AGP columns)	Easy setup Low cost Relative low sample consumption Minimal hands-on preparation or intervention	Binning/relative ranking not absolute percentage binding
96-Well Fluorescence	Rapid Automated Easy setup K_d	Monitoring binding only to tryptophan residue of Human Serum Albumin and α-AGP May miss other binding interactions
Biosensor (Biacore)	Semiautomated % Binding and K_d	Higher time commitment and cost for setup More sample may be required Skilled operator required Solubility issues may be encountered
TRANSIL™ Membrane	Rapid Automated Sensitive % Binding and K_d	Individual proteins or blend of key proteins Not physiological conditions
Capillary electrophoresis	% Binding and K_d Wide dynamic range of K_d Online determination—no sample preparation required	Poor sensitivity Poor reproducibility Compound adsorption to capillary walls Highly acidic compounds problematic
Solid-phase microextraction	% Binding No sample preparation 96-well format	Not commercialized May observe sensitivity limitations and binding saturation for highly bound compounds

throughput via 96-well formats (see below), recent work at Astra Zeneca has shown that compound pooling may be utilized to increase the number of compounds assayed in a single experiment to as many as 10 [15]. On the basis of the high protein content in plasma (30–50 mg/mL HSA, 0.75 mg/mL α-AGP) relative to analyte concentration (typically 10–1000 ng/mL), compound pooling offers a simple but powerful mechanism to quickly obtain screening information. However, if multiple compounds bind specifically to α-AGP, pooling may introduce erroneous results compared to single-compound incubations.

Three different 48- or 96-well commercial equilibrium dialysis devices have been introduced since 2005. In the HT-Dialysis apparatus, eight dialysis membranes are vertically mounted between Teflon spacers that comprise the individual wells [14]. Owing to the vertical design, 96-well liquid handling devices can be used for automated buffer and plasma transfers. The device itself is reusable, with the membrane serving as the primary device consumable. The 96-well Dispo-Equilibrium Dialyzer proposed by Kariv *et al*. [17] utilize a single horizontally mounted dialysis membrane contained in a single-use unit. Use of liquid handling automation for sample handling is still feasible with the horizontal design, albeit with user intervention. The 48-sample Pierce Rapid Equilibrium Dialyzer (RED) system [16] offers a faster equilibrium time (1–2 h) because of a large surface area/volume ratio.

In a typical equilibrium dialysis experiment, blank phosphate buffer solution is added to one side of the dialysis membrane in each well and an equal volume of analyte-spiked plasma to the other side. The equilibrium dialysis device should be incubated at 37°C in a CO_2 atmosphere or with a strong phosphate buffer [17–19] to minimize plasma pH shifts over the experimental time course. Work done by Kochansky *et al*. has shown that pH may increase to 8.7 over 22 h incubation under ambient conditions [18]. This pH shift may alter the protonation state of the analyte thus affecting plasma protein affinity. Once the incubation is complete, individual aliquots are taken from both the plasma and buffer sides and quantitatively assayed by either LC-MS/MS or radiometry.

The analyte free fraction (f_u) is calculated using the following equation. This equation assumes that error added because of a Donnan equilibrium volume shift [20,21] is within the error of the assay method, and therefore negligible. The volume shift may be observed with long equilibrium times such as 24 h; however, compounds or devices that allow a rapid equilibration time of 4 h are unlikely to result in a significant shift.

$$f_u = \frac{C_{buffer}}{C_{plasma}}$$

where C_{buffer} is the unbound compound concentration in buffer after dialysis and C_{plasma} is the postdialysis plasma concentration.

The percent of drug unbound (free) and bound to protein are calculated as follows:

$$\% \text{ Free} = f_u \times 100$$

$$\% \text{ Bound} = (1 - f_u) \times 100$$

A potential problem and source of high variability with equilibrium dialysis is protein breakthrough from the protein-rich plasma side to the aqueous side of the dialysis cell, causing false elevation in the buffer compound concentrations. This can be

monitored by taking an aliquot of any remaining buffer, adding acetonitrile to precipitate the protein, and checking the solution for visible particulates. Use of a membrane with appropriate molecular weight cutoff (10 kDa minimum) will help prevent this problem.

During equilibrium dialysis, an equilibration time of 24 h is recommended for the sake of efficiency. However, scientific judgment can be utilized to reduce this time to 4, 6, or 8 h. Equilibrium dialysis devices that allow for agitation may allow for a faster achievement of equilibrium. For definitive data, it is important to assess at least two sequential time points to ensure that equilibrium has been reached. Analytes can be lost during equilibrium dialysis by nonspecific binding to the membrane and apparatus, decomposition of the compound, or solubility issues. Assessment of this loss can be performed in a separate recovery experiment or during determination of equilibration time. As equilibrium dialysis is a comparative technique, the two halves of an individual well should exhibit similar non-specific binding or recovery issues, thereby minimizing negative impact.

23.3.3 Ultracentrifugation

Ultracentrifugation of plasma results in a protein pellet and an essentially protein-free supernatant [22–25]. Plasma is spiked with the test compound. After centrifugation at 200,000 relative centrifugal force (RCF) for 18 h or 550,000 RCF for 4 h at 37°C, the protein bound material is pelleted with the proteins. The unbound drug material remains in the supernatant, which can be analyzed by radiometric or LC-MS techniques depending on whether the test article is radiolabeled. Generally, it is advisable to take a fraction of the supernatant from the center of the tube to avoid lipids, which may lie at the surface, and any drug concentration gradients that may occur as a result of the centrifugation. This technique largely avoids the problem of nonspecific binding to the apparatus; however, it is a low throughput method requiring an ultracentrifuge. For compounds with high plasma protein binding observed with equilibrium dialysis or ultrafiltration, it can afford a suitable alternative because it may reduce the impact of confounding factors such as solubility and nonspecific binding. The main disadvantage of ultracentrifugation relates to the presence of trace amounts of lipoproteins in the "protein-free" supernatant and highly bound drugs may be associated with these macromolecules. Therefore, compounds measured as free drug in the supernatant may actually be bound, and this will lead to an artificially high free fraction determination.

Buffer controls are used to test for nonspecific binding to the tubes. One option is to test at a concentration that is equivalent to 1% (assuming a 99% protein bound compound) of the lowest concentration tested to mimic the free fraction of a highly bound compound.

The equation used for calculations is as follows:

$$\%\ f_{\mathrm{up}} = \frac{\text{Measured drug concentration post ultracentrifugation}}{\text{Measured drug concentration pre ultracentrifugation}} \times 100$$

23.3.4 Detection—Radiometric or LC/MS/MS

Radiometric detection had been the classic method of quantitation for plasma protein binding until the invention and broad use of LC/MS/MS methodology. The use of

tritium or carbon-14 labeled materials allow a facile determination of absolute recovery from the various plasma-protein-binding devices and a ready measure of drug amount. A potential drawback of radiometric determination occurs for very highly bound drugs and relates to the purity of the label. When the percentage of radiochemical purity of the radiolabel approximates the percentage of plasma protein binding, it is possible that the unbound material measured is actually impure rather than drug material. This puts the results into question. For this reason, the use of LC/MS/MS may afford a more accurate measure for very highly bound drug candidates. The use of standard curves to ensure quantitative measure and comparison to appropriately spiked control samples will allow quantitation for the purpose of recovery calculations.

Historically, highly bound compounds were considered to be those with binding of >95%. With current techniques (either highly pure radiolabel or the use of LC/MS/MS), accuracy within this range has improved considerably and the current limit is considered to be ~99%. If one considers a 10 μM concentration, 1% unbound material that represents 100 nM, a reasonable limit of detection for LC/MS/MS methods.

23.4 EMERGING TECHNIQUES

Exploratory work continues to investigate new opportunities to improve plasma protein binding beyond the previously described high throughput traditional approaches. These so-called "Emerging Techniques" employ methodologies ranging from chromatography, CE, spectroscopy, biosensors, microextraction, and model artificial membranes. Table 23.1 describes the relative advantages and issues for traditional and screening *in vitro* plasma-protein-binding techniques.

23.4.1 Chromatographic Methods

Significant effort has been invested over the years in assessing plasma protein binding through chromatographic methods based on HPLC columns containing the most abundant plasma proteins, HSA and α-AGP [26–33].

Through the usual chromatographic partitioning processes, binding can be correlated with chromatographic retention time. In a typical experiment, the capacity factor (k') of each compound is calculated based on its retention time, using the following formula:

$$k' = \frac{t_r - t_m}{t_m}$$

where t_r is the retention time of the analyte and t_m is the retention time of an unretained compound, also known as the *column void volume*, and t_m can be assessed using glucose, cesium iodide, or ammonium nitrate. From the k' values, $(k'/k' + 1)$ is calculated and then plotted as a function of the known plasma protein binding, as shown in Fig. 23.3. After the correlation plot is generated, the plasma protein binding of unknown compounds can be calculated based on their retention time on the albumin or α-AGP column. In general, the factor $k'/k' + 1$, rather than k', shows better linear correlation with plasma-protein-binding values. As shown in the Fig. 23.3, compounds can be binned into low, medium, and high protein-binding categories, to aid in screening. When first establishing the method in one's laboratory, a series of compounds with

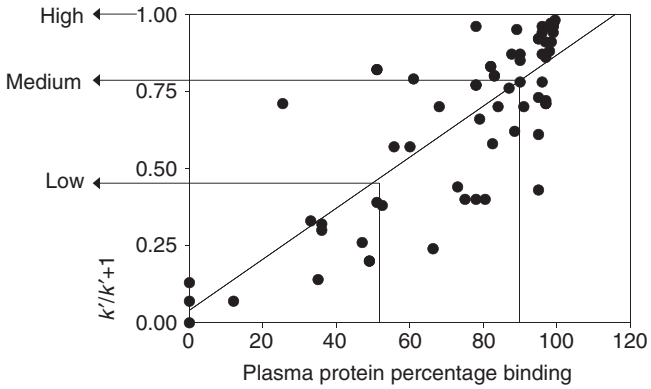

Figure 23.3 Binning of low, medium, and high plasma protein binding by chromatographic retention.

known plasma-protein-binding values should be chromatographically analyzed using an HSA column. In addition, an α-AGP column may be used in series or parallel. The degree of plasma protein binding can be either found in the literature [34,35] or obtained by comparative ultrafiltration or equilibrium dialysis.

Recently, both Hage and coworkers and Wainer and coworkers have published reports on the use of in-house prepared HSA affinity microcolumns for plasma-protein-binding determinations [36–38]. Both frontal affinity and zonal elution chromatography have been utilized, each with its own advantages and disadvantages. The primary stumbling block to widespread application of these techniques within the pharmaceutical industry is the commercial availability of the HSA microcolumns.

In practice, most pharmaceutical laboratories screen only against the HSA column, accepting the risk that binding α-AGP or other less-abundant serum proteins may be completely underestimated. This method generally performs better for compounds exhibiting low to moderate plasma protein binding rather than high (>95%).

23.4.2 Capillary Electrophoresis Methods

The application of CE to the measurement of plasma protein binding represents a logical mechanism to leverage the inherent ability of CE to separate analytes based on electrophoretic mobility. CE techniques utilized for this purpose essentially examine the difference in analyte mobility in bound and unbound states to various proteins. Affinity capillary electrophoresis (ACE) techniques have been utilized for relative rank ordering of compound libraries versus various protein receptors [39], which can be measured during the separation step or against a solid-phase ligand support. This methodology offers the advantages of small sample volume requirements, relatively rapid measurements under equilibrium conditions, and at pH 7.4 "near physiological conditions," similar to traditional techniques such as equilibrium dialysis. In a study conducted by Ishihama *et al*. [40], this technique was applied to six anionic drugs. Their binding to HSA correlated with values obtained by ultrafiltration over a range of 78.7–99.9% binding, with typical reproducibility <10% CV (coefficient of variation). However, the required drug concentration in solution was 20 μM.

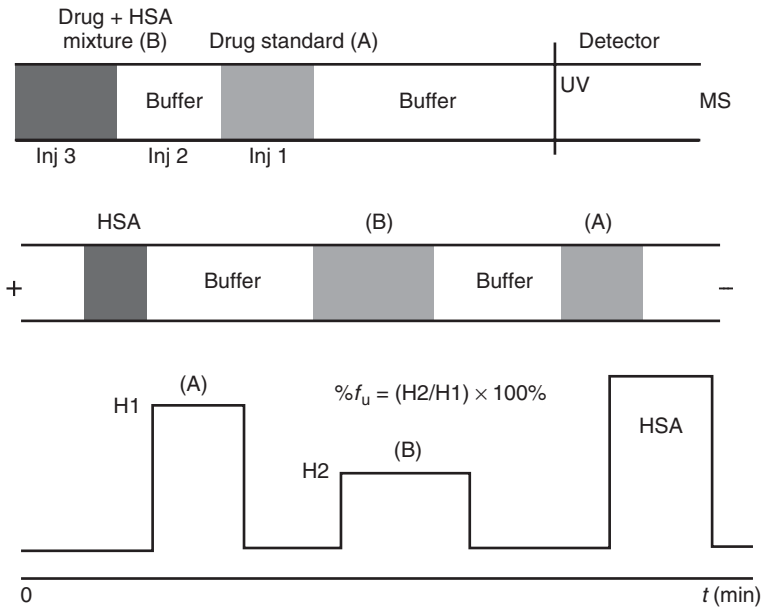

Figure 23.4 Illustration of single-run measurement of protein binding by high performance frontal analysis capillary electrophoresis. H1 and HB are the peak plateau heights of the drug standard and the drug resolved from the drug–protein mixture, respectively. $\%F_u$ is the unbound free fraction of drug to specific protein.

Higher sample volume online approaches can be leveraged by combining frontal analysis with CE. Figure 23.4 shows a schematic of this process, described as frontal analysis capillary electrophoresis (FACE) An injection of neat analyte solution is made, followed by a buffer plug, and subsequently by an injection of analyte + protein mixture. On the basis of binding equilibria as well as relative electrophoretic mobility, the resulting electrophoretogram will contain a free analyte peak, which can be used as a relative standard response against a separate, later eluting peak corresponding to analyte not bound to the protein of interest. Depending on experimental conditions, the unbound analyte peak may or may not be resolved from the protein peak and may instead appear as a shoulder on the larger protein peak. CE is most commonly coupled with UV detection and was leveraged for plasma-protein-binding experiments in a study of protein–drug binding constants for HSA and α-AGP [41,42]. More recently, commercial interfaces to mass spectrometers have been developed and utilized for plasma-protein-binding determinations [43]. The analyte concentrations required can be decreased from the $\geq$1 mM to $<$20 μM range. Better correlation of percentage binding values were observed with the plasma was diluted to 10% plasma in buffer. In addition, a dependence of percentage binding on time interval between sample injections was observed. It was hypothesized that because of significant lower concentrations for MS detection compared to UV resulted in differential adsorption of the analyte to the capillary walls, rather than saturation at high concentration. This technique provides rapid screening values but has not yet matured to a stage where precise percentage binding values can reliably be obtained, particularly for highly bound compounds.

23.4.3 Spectroscopic Methods

The 96-well fluorescence approach relies on the detection of intrinsic fluorescence of the tryptophan residues of HSA and α-AGP, the primary plasma protein constituents [44]. As drugs bind to the protein, the fluorescence signal is quenched as a function of analyte concentration. The analyte's dissociation constant, k_D, can then be calculated. Although this method is readily automated, its universality is questionable, since tryptophan residues reflect only a portion of the potential binding sites to both α-AGP and HSA.

23.4.4 Biosensors—Surface Plasmon Resonance

Another high throughput technique for plasma protein binding utilizes the Biacore™ surface plasmon resonance (SPR) technology as shown in Fig. 23.5 [45–48]. Proteins of interest are immobilized onto a gold foil. The drug is exposed to the proteins, binding occurs in real time, causing a change in refractive index of a light beam. The kinetics of both the on-rate and off-rate is captured in a sensorgram. After determining the dissociation k_D values from sensorgrams of multiple solutions containing several concentrations of drug, the corresponding percentage binding can be calculated. The binding to both albumin and α-AGP is combined to give the overall plasma protein binding for these two proteins. If binding to proteins other than albumin or α-AGP are important, the plasma protein binding will be underestimated. The need to purchase Biacore SPR instrumentation as well as instrument and chip synthesis proficiency can be potential limitations.

SPR has the potential to become a very high throughput technique since the use of microfluidics and the generation of protein arrays on the chip allow for highly multiplexed approaches [49]. Although this technique requires a significant amount of time and expertise to establish, once operational, it can provide extremely high throughput results. In addition, the potential to address the critical gap of very high bound plasma-protein-binding determination is an intriguing future prospect.

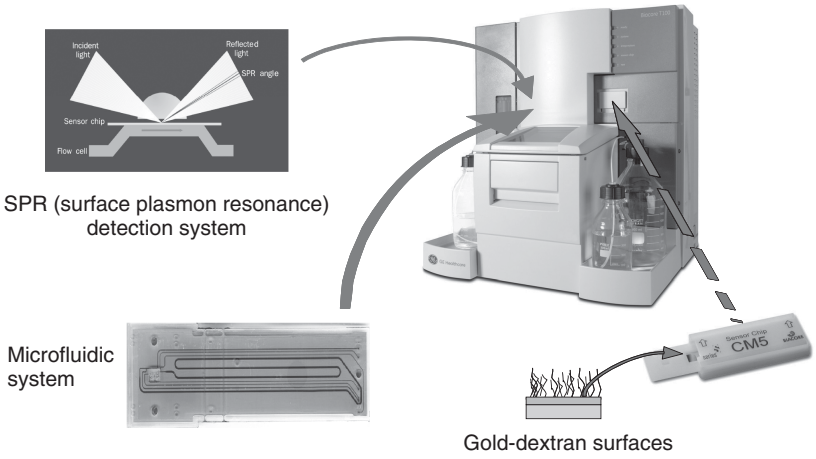

Figure 23.5 Experimental approach and equipment required for Biacore™ analysis of binding by surface plasmon resonance.

23.4.5 Solid-Phase Microextraction

Solid-phase microextraction (SPME), pioneered by Janusz Pawliszyn and coworkers [50], represents one of the more recent developments in general analytical sample preparation. This methodology relies on the extraction of the analyte of interest from liquid or gaseous media onto a solid support, generally a polymeric coating on a metal rod. The microextraction probe is engineered in a manner to enable maximal surface contact and ultimately analyte adsorption. This technique has been utilized to determine plasma protein binding for a set of pharmaceutical compounds including ibuprofen, warfarin, verapamil, propanolol, and caffeine with low, intermediate, and high protein-binding properties. Good correlation was observed between literature and SPME-derived protein-binding values. Figure 23.6 shows a schematic of the SPME experimental design for protein binding. Several variables were optimized including choice of fiber material, pH control mechanisms, and plasma dilution with phosphate buffer saline (PBS). Plasma dilution serves a very useful purpose of allowing highly bound (>99.9%) drugs to be reliably quantitated by decreasing the amount of plasma proteins and correspondingly, the percentage bound. Although commercial devices for this technique are not yet available, exciting possibilities for 96-well parallel extraction designs can be easily envisioned.

23.4.6 TRANSIL™ Membrane and Protein Beads

The TRANSIL™ membrane beads are used to replace erythrocytes [51] in partitioning assays and are uniquely suited to plasma-binding determinations for highly bound or low aqueous solubility drugs [52,53]. In this assay, the analyte is incubated together with plasma and the membrane beads for 30 min. By determining drug concentrations in plasma after incubation, the assay circumvents issues with compounds with poor aqueous solubility. This assay monitors the decrease in analyte plasma concentration rather than the appearance of analyte in another phase (buffer). As essentially an indirect measurement, the need for ultrasensitive quantitation for highly bound drugs is avoided.

To determine the affinity of drugs to plasma, the change in affinity to the membrane beads, induced by changing the plasma concentration, is measured. The fraction

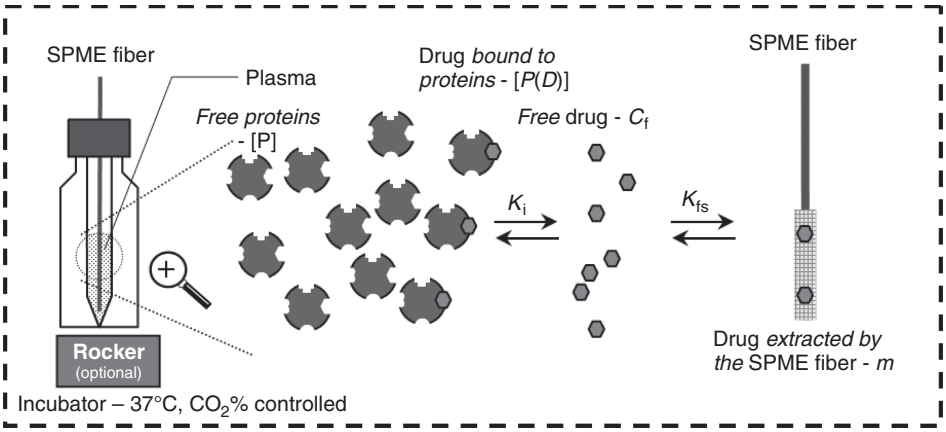

Figure 23.6 Schematic representation of experimental setup for the determination of plasma protein binding by solid-phase microextraction (SPME).

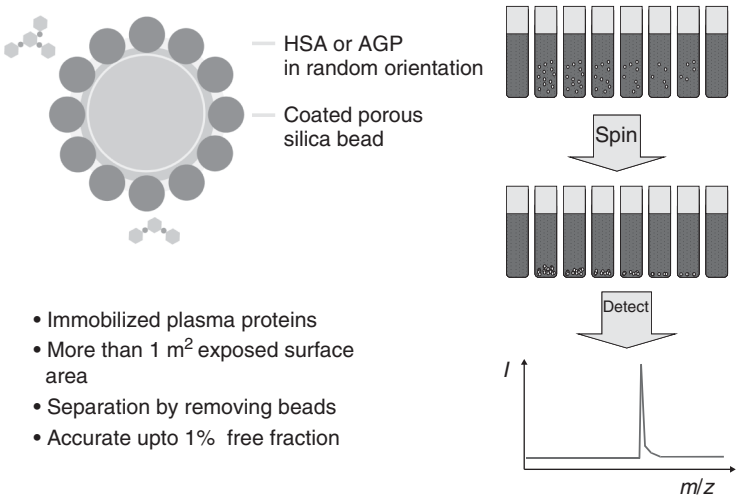

Figure 23.7 Illustration of TRANSIL™ beads and approach for measuring binding using beads embedded with plasma proteins.

bound is calculated from linear regression of membrane affinity (M_A, Y) measurement data obtained from different plasma dilutions (D_{plasma}, X). The calculated slope α and intercept M_{A0} are then inserted into the following equation:

$$f_b = 1 - \frac{1}{1 + \alpha M_{A0}}$$

This approach allows accurate measurement of free fractions of 0.01 or lower.

A rapid technique for predicting plasma binding from affinities (K_d) to HSA and AGP is provided by the TRANSIL protein bead assay system based on HSA and α-AGP mobilized on silica beads. The beads are suspended in a solution of drug and after a few minutes, centrifugation is performed to separate the beads as shown in Fig. 23.7. Versions of the assay exist for HSA, AGP, rat albumin, and HSA and AGP mixed together at a ratio of 24:1 to mimic plasma conditions. Quantitation of analyte remaining in the supernatant may be quickly and reproducibly measured by radiometry or LC/MS/MS, as appropriate

23.5 IMPACT OF HIGH (UNDETERMINABLE) PROTEIN BINDING ON COMPOUND DEVELOPABILITY

It is common that highly lipophilic drugs may exhibit very high plasma protein binding, in excess of 99%. Owing to analytical limitations, the accuracy of determinations above 99% bound are not reliable when radiometry or LC/MS/MS is involved. For radiometric determination, the radiolabel must be >99% pure in order to be confident that the free fraction observed is because of drug and not impurity. Radiolabel with this degree of purity may be very difficult to obtain and is well above the purity typically required for other purposes such as excretion or metabolite profile studies

where 95% radiochemical purity is sufficient. For LC/MS/MS, a 99% plasma protein bound (PPB) compound would yield 1% free, leading to 10 nM LOQ for a study conducted at a typical 1 μM starting concentration. This LOQ is readily measurable for most NCEs (new chemical entities) but not necessarily with the requisite precision and accuracy to report greater than unit percentage binding values with confidence. Most LC/MS/MS methods rely on relatively simple generic sample extraction methods (protein precipitation) as well as chromatographic and mass spectrometric conditions. The emphasis of method development at the time protein-binding assays are conducted is generally on speed (turnaround time), not exquisite sensitivity. The most pragmatic approach taken, if LC/MS/MS sensitivity cannot be readily improved, is to simply report >99% binding and move on to the next experiment.

The inability to accurately determine the plasma protein binding is an issue because it is the free fraction that is important for the determination of PK/PD and safety margins. Above 99% binding, the corresponding free fraction is 1% or less and differences become pronounced. For example, the difference in free fraction between 99.4% and 99.8% is a factor of 3; 0.6% versus 0.2% free. Analytical methods that provide inadequate sensitivity and corresponding LOQ serious hamper decision making. Inability to precisely and accurately determine percentage binding values for very highly bound compounds seriously limits accurate PK/PD interpretation from preclinical species or prediction of necessary dosing regimens that will provide efficacious doses in the clinic.

In situations where the PPB is very high, one recommended approach is to conduct the experiment with plasma that is diluted to 10% in phosphate buffer solution. The undiluted plasma result can then be back calculated to plasma binding by the equation [54], where D and $F_{u,measured}$ represent the fold dilution of plasma and unbound fraction measured with diluted plasma, respectively.

$$\text{Undiluted } f_{up} = \frac{1/D}{\left[\left(1/F_{U,measured}\right) - 1\right] + 1/D}$$

23.6 CONCLUSIONS

Plasma protein binding is clearly an important ADME parameter required to understand drug efficacy and possibly toxicity. Recent progress in automation as well as increased routine performance of the analytical instrumentation has enabled a greater number of compounds to be assayed earlier in discovery, often at a high enough quality level such that repeated determinations later in development are not needed. Significant challenges still remain unsolved, particularly in cases of high protein binding with low exposure levels that fall below the normal LOQ. As technology improves even further, we can expect more opportunities to understand and leverage this important information to an even greater extent.

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