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Review

Ultrafiltration Method for Plasma Protein Binding Studies and Its Limitations

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Abstract: Plasma protein binding plays a critical role in drug therapy, being a key part in the characterization of any compound. Among other methods, this process is largely studied by ultrafiltration based on its advantages. However, the method also has some limitations that could negatively influence the experimental results. The aim of this study was to underline key aspects regarding the limitations of the ultrafiltration method, and the potential ways to overcome them. The main limitations are given by the non-specific binding of the substances, the effect of the volume ratio obtained, and the need of a rigorous control of the experimental conditions, especially pH and temperature. This review presents a variety of methods that can hypothetically reduce the limitations, and concludes that ultrafiltration remains a reliable method for the study of protein binding. However, the methodology of the study should be carefully chosen.

Keywords: ultrafiltration; plasma protein binding; non-specific binding; limitations

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1. Introduction

Plasma protein binding (PPB) is a reversible process that plays a critical role in drug therapy, influencing both the pharmacokinetic and pharmacodynamic properties of drugs. Binding to plasma proteins is important, especially in the case of drugs with a high affinity to plasma proteins (>99%) and in polytherapy treatments because of the displacement interactions that can occur, which lead to a high risk of adverse drug reactions. Evaluating the degree of PPB is required in the early stages of drug discovery and development, and the existence of an analysis method that can provide accurate and relevant results is essential [1–3].

Regarding the binding to plasma proteins, the interaction with a specific site is determined, on the one hand, by the chemical structure of the drug, the pKa of the substance, and the pH of plasma; on the other hand, the type of protein involved (albumin, alpha-1 acid glycoprotein, lipoproteins, and globulins) is also important. In the case of albumin, of the eight binding sites, the most important are the Sudlow I site (warfarin site) and the Sudlow II site (diazepam site) [1]. Site I forms a pocket of hydrophobic chains that

contain basic groups in the area of the portal of entry [4]. Binding to site I is reversible, and is achieved by electrostatic bonds (for acidic substances, such as NSAIDs or coumarin anticoagulants, intensely ionized at a plasma pH of about 7.4). For this type of substance, the fraction of binding is significant (98–99%). Moreover, the binding is saturable and sensitive to competition with other drugs, which may have important clinical consequences: the increase in the free fraction and the pharmacodynamic effect with risk of subsequent overdose by displacement interactions; the consistent binding moiety serves as a reservoir: as the free molecules leave the intravascular space by simple diffusion, the equilibrium shifts toward the detachment of the drug–albumin molecules.

Site II is similar to site I, but smaller in size, having a polar gateway with affinity for hydrophobic/lipophilic compounds, which possess an electronegative area under the effect of blood pH [4]. Substances that bind non-specifically to albumin (e.g., digoxin) are not affected by the risk of displacement interactions because the binding capacity of albumin is much higher than that obtained at therapeutic concentrations. Alpha-1 acid glycoprotein binds preferentially to basic or neutral molecules (not ionized in the plasma), and since it is an acute-phase protein synthesized in the liver, the pro-inflammatory status can directly influence the bound fraction of the studied drugs [1].

Many methods have been proposed for the study of PPB, including: equilibrium dialysis (ED), ultrafiltration (UF), ultracentrifugation, gel filtration, partition method, spectroscopy, calorimetry, chromatographic techniques, capillary electrophoresis, surface plasmon resonance, etc. [1–8]. However, regarding drug-development, only ED and UF gained widespread acceptance [6,9].

Both UF and ED rely on the physical separation of the unbound and bound fractions of a drug through a semipermeable membrane, followed by the quantification of the free fraction using a suitable analytical technique (usually HPLC-UV or HPLC-MS).

Although ED is considered to be the gold standard, UF is becoming increasingly popular due to its main advantage represented by the short analysis time [1,4]. UF is considered to be one of the least time consuming and easiest to apply methods for the determination of the unbound fraction of a drug, having the potential to be used during clinical monitoring.

The principle of the UF method is the separation of small volumes of protein-free phase by applying a centrifugal force to a solution containing both proteins and the substance of interest, located in the upper compartment of a special UF device. The UF device is separated into two compartments by a semipermeable membrane, which has different molecular weight limits for protein filtration [1,6,8]. After separation by centrifugation, the free drug concentration is determined from the protein-free ultrafiltrate located in the lower compartment of the UF device.

Beside its advantages (speed, simplicity, obtaining accurate and quantitative data, and approximation of physiological conditions), UF also has some disadvantages that may limit its use if not corrected [1,10]. The main disadvantage of the UF technique is the non-specific binding of substances to the semipermeable membrane and the compartment of the UF device, which limits the study of PPB for compounds with high lipophilicity using this particular method [1,4,6,8]. Other limitations of the method to be considered are: the sieve effect, the Gibbs-Donnan effect, protein leakage, the need for rigorous control of pH and temperature [1], and the effect of volume ratio of the ultrafiltrate [11].

Due to the constant interest regarding optimization of the UF method for PPB studies, many research papers describe a variety of methods that can hypothetically reduce the limitations, some of which will be discussed further.

2. Non-Specific Binding

The biggest limitation of the UF technique as a study method for the binding of substances to plasma proteins is their non-specific binding (NSB) to the filter membrane and to the material of the UF device, which provides NSB sites due to their characteristic charge and polarity [12,13]. When the analyte shows a high degree of NSB, the concentration

of the free fraction determined from the ultrafiltrate differs greatly from the actual free concentration in the sample under analysis, thus leading to inaccurate results. This is a growing concern due to the fact that in recent years, more and more lipophilic compounds have been developed, which are adsorbed to a large extent and are associated with a higher degree of NSB.

The main parameters that influence the binding of drugs to the filter membrane are the degree of lipophilicity and their molecular mass. The filter material is also important, as its composition modulates the types of interactions. In the case of drugs with marked lipophilicity, adsorption to the filter membrane is very likely, and pre-saturation of the filter may be a solution to avoid NSB, but only if it is performed with a labeled ligand (to avoid errors caused by desorption or competition for binding with the drug that otherwise cannot be quantitatively assessed). The presence of ionizable groups on the surface of the membrane can be a limiting factor in the UF of ionized drug molecules.

With regard to molecular weight, its increase (M > 500) may produce a molecular sieving effect, which may lead to lower drug concentrations in the ultrafiltrate than the expected value [13].

Several possibilities have been proposed to eliminate or lower the degree of NSB.

One of the methods described in the literature as having the potential to reduce NSB of substances consists of the pre-treatment of the filter units with a solution of Tween 80 [13]. This non-ionic surfactant is applied to the filter membrane in order to reduce potential hydrophobic interactions. In these studies, which were performed on analytical solutions prepared in phosphate buffer, it was observed that the degree of NSB modulation was dependent on the concentration of the Tween solution applied and on the concentration of substance in the sample solutions. Thus, the higher the concentration of Tween 80 solutions used, the lower the degree of NSB observed, and the lower the concentration of the substance in the samples, the higher the degree of NSB. Other studies showed a significant reduction in NSB following a pre-treatment of the filter units with Tween 20 solutions [14]. The chosen working methodology, the characteristics of the UF devices used, and the laboratory equipment are highly important in reducing the degree of NSB by this method.

Although efficient in some cases, the pre-treatment with Tween solutions to reduce NSB was not found to be effective for all compounds, and as a result, a pre-treatment with benzalkonium chloride was proposed as a possible and alternative way to further reduce NSB based on its capacity to prevent potential ionic interactions between basic compounds and the filter membrane [13,15]. Studies have shown, however, that although this alternative method is effective for basic compounds, it increases the degree of NSB in the case of acidic compounds and has no effect in the case of neutral and hydrophilic compounds.

Considering these results, a selection criterion for choosing the pre-treatment agent of the UF tubes can be stated as follows: Tween 80 reduces NSB of acidic and neutral compounds; benzalkonium chloride reduces NSB of basic compounds [13]. However, the pre-treatment of the UF units with Tween solutions raises some concerns due to having been shown to interact with plasma proteins [16,17] and to interfere with the process of drug binding (as observed with docetaxel, which is extensively bound to proteins) [18].

To lower the impact of NSB on the experimental results, the degree of NSB of substances was usually determined using solutions of the analyte in phosphate buffer saline (PBS), which were subjected to UF. Based on the results, a correction factor was then applied to the results obtained after a sample analysis [6]. However, subsequent studies conducted by Wang and Williams suggested that this is not a feasible alternative, noting that the behavior of some compounds regarding NSB differs when incubated with the UF device in plasma compared to PBS [12]. This is due to serum proteins being adsorbed on the surface of different types of materials, leading to the blocking of most NSB sites. These studies had an approach based on mass balance, and showed that the limitation given by the NSB can be corrected, even in the case of compounds with increased lipophilia, because NSB sites are inactivated in the presence of plasma. However, there is another concern regarding

NSB that may occur in the ultrafiltrate compartment, where, in the absence of plasma, the risk of NSB increases [6].

Taylor and Harker proposed a modification of the UF technique to eliminate NSB of lipophilic compounds, consisting of the simultaneous UF of the sample being subjected to analysis with a control plasma sample [19]. After centrifugation, the units containing the retentates are exchanged between paired filter units and recentrifuged, thus obtaining two reconstituted samples, one containing the unbound drug, and the other containing the bound drug. Using this modified method of the classical UF technique, NSB was successfully eliminated for some corticosteroid compounds.

3. Effect of Volume Ratio

Following the traditional UF, a high volumetric ratio, which cannot be properly controlled, is usually obtained between the ultrafiltrate and the sample solution [11]. It was thought that this volume ratio may affect the protein binding equilibrium and influence the analysis of the free fraction.

During UF, the aqueous component of the plasma, which contains the free drug molecules, is forced to pass through the semipermeable membrane due to the pressure gradient. As a result, there is a transient decrease in the free drug concentration, while the protein and protein–drug complex concentrations increase for the remaining sample fraction in the upper compartment of the UF device. Consequently, the initial protein binding equilibrium is considered to be disturbed while a new equilibrium is being established.

Some researchers have recommended the volume of ultrafiltrate to be less than 35% of the plasma volume [20], others have recommended it to be less than 20% [21], or even less than 10% [1], to minimize the disturbance of the protein binding equilibrium. Subsequent studies, however, stated that the disturbance of the initial equilibrium due to increased protein concentration in the upper compartment of the UF units is only a misunderstanding of the process that occurs. The explanation is that during UF, balanced sample portions are removed, which do not affect the drug–protein equilibrium above the filter membrane. These data are presented both theoretically and experimentally in a study conducted by Nilsson, in which the same results were obtained for ultrafiltrate volumes between 16% and 47% of the initial plasma volume [6]. Even so, results obtained in some experiments with very large volumes of ultrafiltrate must be considered (up to 80% of the sample volume) in which an increasing concentration of the free drug fraction with an increasing volumetric ratio between the ultrafiltrate and the sample solution was observed [11]. However, this was also considered to be a consequence of the fact that in order to obtain such volumes of ultrafiltrate, it is necessary to apply a very high centrifugal force, which can lead to unwanted protein leakage and increases in temperature, factors that may also affect the experimental results [6].

Because the classical UF method and devices do not allow for such rigorous control of the ultrafiltrate volume and are susceptible to many factors, some researchers have suggested and demonstrated that a hollow fiber centrifugal ultrafiltration (HFCF-UF) is a more accurate alternative for the determination of the unbound drug. HFCF-UF is a more robust method compared to the classical UF, being less affected by experimental conditions and allowing for an exact control of the volume ratio [11,22–24].

HFCF-UF is a more reliable approach, especially in the case of samples with lower serum albumin concentrations, because the influence of the volume ratio on the experimental results was also shown to be dependent on this parameter, being greater with decreasing concentration [25,26].

4. Influence of Experimental Conditions

The experimental conditions in PPB studies are of great importance as they may greatly affect the results of the analysis [27,28]. Among these, the filter device, membrane type, pH, temperature, and relative centrifugal force (RCF) must be considered.

The Millipore Centrifree UF devices (Merck, Germany) are used in most plasma protein binding studies, but good results using modern devices initially designed for protein or RNA solution concentration (for example, Vivaspin, Vivacon, and Nanosep) were also reported in a study designed by Kratzer et al. [27]. However, these devices require validation to the Centrifree devices, which are used as a reference. Although they are able to be used at a high centrifugal force, in the case of PPB studies, this is not applicable due to the occurrence of the pressure effect. This effect leads to changes in the permeability of the membrane for drugs and water depending on the RCF applied and the molecular weight of the substance. Experiments have shown that the measured free drug fraction decreases as the RCF increases, the effect being more obvious in the case of substances with higher molecular weight. Due to the amplitude of the effect being dependent on the molecular weight of the analyte, it is not possible to establish an optimal value or an upper limit that is generally valid for the RCF. However, the Centrifree user manual recommends a maximum value of 2000$\times$ g. Very close attention should be paid when choosing the UF device because different devices can lead to different experimental results for the same compound (as described for imatinib using Amicon vs. Centrifree devices) [15].

Regarding the centrifugation time, the studies performed did not show a significant influence on the results obtained [27,29].

The membrane type of the UF device is also a variable that should be considered when conducting PPB studies because of the possibility of analyte absorbance. Studies have shown that different results can be obtained for the same compound when using devices with a polyethersulfone membrane compared to cellulose; in addition to this, depending on the membrane, the analyte's behavior can vary greatly [27,28]. Generally, devices with a regenerated cellulose membrane are preferred. However, depending on the compound, good results can also be obtained using other membranes, so this remains a topic of debate.

Another important parameter for PPB studies is the pH, which has a significant influence due to the binding of substances to plasma proteins being dependent on its value [6,30–32]. It has been observed that in the case of basic compounds, which normally bind to alpha-1-acid glycoproteins, the free fraction decreases with an increase in the pH due to an increase in the non-ionized fraction. Whereas in the case of acidic compounds, which are mostly bound to albumin, it has been observed that an increase in the pH can either result in an increase or a decrease in the free fraction of the drug. These results are thought to be due to the conformational changes that may occur in the structure of albumin. In the case of neutral compounds, PPB is less affected by changes in the pH [30].

Normally, the pH of the circulating blood has a value between 7.35 and 7.45 maintained especially by the bicarbonate buffer system [6,27]. After collecting the blood, the pH value increases, reaching values even higher than 8, due to a continuous loss of carbon dioxide that occurs during the storage and preparation of the sample. Therefore, restoring the pH to its physiological value is essential.

Regarding the temperature, studies have shown there is a decrease in the free fraction of the drug with the temperature falling below the physiological value of 37 °C, and studies conducted at room temperature can give results of even 50% of the unbound fraction determined at 37 °C [6,27].

Considering all these aspects, a rigorous control of both the pH and temperature is required. The values of these parameters must always be adjusted to physiological conditions to obtain relevant results.

5. Conclusions

Although UF has its limitations, there are a various number of ways to correct, reduce, or even eliminate them, making it possible to use the method even for more challenging compounds. UF still remains a reliable method for PPB studies, but the methodology of the study should be carefully chosen for each analyte, and very close attention should be paid to the processing and preparation of the samples and experimental conditions.

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