Challenges regarding analysis of unbound fraction of highly bound protein antiretroviral drugs in several biological matrices: lack of harmonisation and guidelines

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The unbound drug concentration in plasma is usually considered the only active fraction; thus the binding of a drug to a protein limits its pharmacological actions. This is of special importance for those highly bound drugs. Therefore, binding studies can be of great utility for those drugs where relationship between free and total drug concentration is variable among patients, or it can be altered by some condition or disease, or even by interactions with other drugs. However, there is a lack of validation guidelines for the determination of unbound concentrations. Antiretroviral drugs (ARVs), protease inhibitors (PIs), efavirenz and nevirapine are highly bound to proteins. Here, we present a review on the overall methods for the study of unbound fractions of highly bound plasma protein ARVs. We also provide a critical evaluation of the methods applied, their differences and the main points to be controlled and validated.

Introduction

Protein binding greatly influences the ADME processes for highly plasma bound drugs [1]. There are two main proteins involved in plasma binding: human serum albumin (HSA) and α1-acid glycoprotein (AAG). HSA, a 66 kDa globular protein, is the major protein component in plasma (~60%). There are two main binding sites on HSA with a slight preference for acidic drugs. By contrast, AAG, a 38–48 kDa protein, mainly binds basic and neutral drugs. As an acute-phase protein, AAG can be elevated in different diseases. In plasma, the unbound fraction is usually considered as the only active fraction, because it is the only one able to pass through membranes to reach tissues and penetrate into cells to exert its activity.

Antiretroviral drugs (ARVs) present large differences in plasma protein binding (PPB). HIV protease inhibitors (PIs) are mostly lipophilic weak basic molecules, highly bound to plasma proteins, mainly to AAG (greater than 85% except indinavir at 60%). The non-nucleoside reverse transcriptase inhibitors (NNRTIs) efavirenz and nevirapine, which are weakly acidic compounds, mainly bind to HSA (greater than 99% and 60%, respectively). Other NNRTIs, and also the nucleoside reverse transcriptase inhibitors (NRTIs), are weakly bound to plasma proteins. Table 1 summarises protein binding and some biochemical properties across the ARVs reviewed.

Therapeutic drug monitoring (TDM) has been proposed for optimising dosing regimens of ARVs [2,3]. However, it is based on total plasma concentration instead of unbound concentration. A small change in the extent of protein binding of highly bound drugs can result in a dramatic effect on the active free fraction. In fact, the relationship between unbound concentrations and unbound fractions is only described as linear at low free concentrations, but not when the binding proteins are saturated [4]. This can occur in some physiological or pathological conditions, such as pregnancy or infections [4,5]. The study of free (unbound) drug concentrations can be of great utility whenever ARV PPB changes

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TABLE 1

Percentage of plasma protein binding (Fb %), molecular weight (MW), partition coefficient (log *P*) and group for the ARVs reviewed

Drug name	Group	MW (Da)	Log *P*	Fb (%)	Refs
Amprenavir	PIs	505.63	2.43	90	[49]
Atazanavir	PIs	704.86	4.54	86	[49]
Darunavir	PIs	547.24	2.82	95	[50]
Efavirenz	NNRTIs	315.68	4.46	99	[49]
Indinavir	PIs	613.36	2.81	60	[49]
Lopinavir	PIs	628.36	4.69	>98	[49]
Nelfinavir	PIs	567.31	4.72	>98	[49]
Nevirapine	NNRTIs	266.12	2.49	60	[49]
Ritonavir	PIs	720.31	5.22	90–98	[49]
Saquinavir	PIs	670.38	2.58	>98	[49]
Tipranavir	PIs	602.21	7.81	99.9	[51]

Abbreviations: ARVs, antiretrovirals; PIs, protease inhibitors; NNRTIs, non-nucleoside reverse transcriptase inhibitors.

occur, improving the TDM approach based on total drug concentrations and contributing to a better individualisation of drug dosage regimen. Recently, Fayet *et al.* [6] demonstrated that the decrease on total lopinavir concentration during late pregnancy was not associated with a decrease in the free concentrations. For this reason, dosage adjustment during pregnancy is not required. This goes against the recommendations of some experts who suggest increasing the lopinavir dosage in the second and third trimesters of pregnancy, which is only based on the decrease of total lopinavir concentrations during late-stage pregnancy. Other evidence of the great utility of unbound concentrations is the arising population of pharmacokinetic models that integrate the contribution of protein concentration to variability on drug concentrations of highly bound drugs. The study of unbound concentrations can also be important because of the potential advantage of high protein binding for ARVs. Protein binding is usually related to an increase in the distribution volume of the drug. As a consequence, this high protein binding can be a potential benefit for the single tablet regimens of ARVs that are currently in development.

The study of PPB includes different steps. Figure 1 gives an overall scheme of the processes. Given a sample, the first step is the separation of the unbound and bound fractions. Several methods are available, including equilibrium dialysis (ED), ultrafiltration (UF), ultracentrifugation, microdialysis and chromatographic methods such as frontal analysis and electrophoresis, among others. Extensive descriptions and reviews of all the techniques used for the separation of unbound fractions of drugs can be found throughout the literature, for example see [7,8]. However, the most-used and -well-accepted techniques from a drug discovery point of view regardless of the drug analysed are ED and UF. After separation, the samples are sometimes submitted for extraction or dilution. Thereafter, a quantification method is applied, either a selective or nonselective analysis. This work is a review of the studies reported for the determination of unbound concentrations of ARVs highly bound to plasma protein. It focuses on a description of the different steps of the overall process, providing a critical evaluation of the different methods, their differences, advantages and disadvantages and the main points to be controlled and validated. Finally, current research and clinical applications are summarised. Table 2 summarises all the steps of the overall process of PPB studies on ARVs.

Separation of unbound fraction

A semipermeable membrane divides two chambers in ED. One compartment contains a buffer solution similar to plasma water

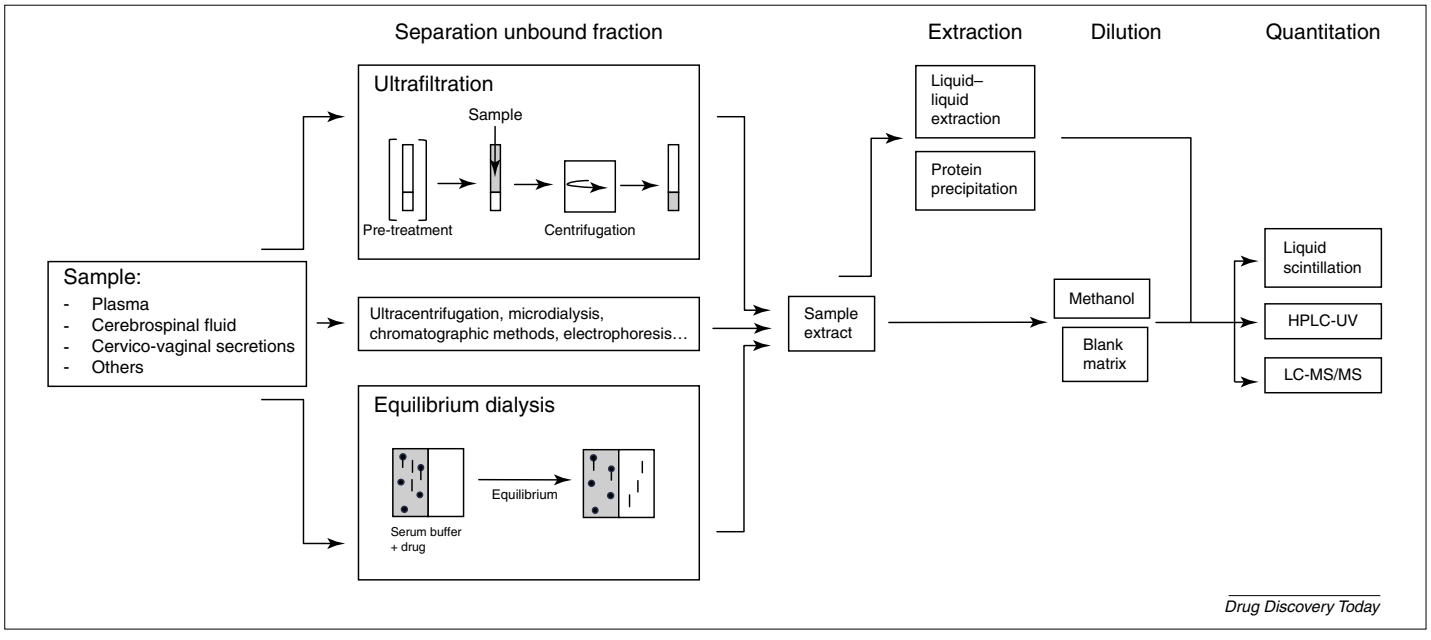

FIGURE 1

Scheme of all the steps involved in the study of plasma protein binding. The left panel shows the kind of sample to be analysed. The first step of the study is the separation of the unbound fraction, usually by ultrafiltration or equilibrium dialysis. After its separation, the unbound fraction is either extracted (liquid–liquid extraction or protein precipitation) or directly diluted with methanol or its blank matrix. Then, the unbound concentrations are quantitated.