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Global analytical strategy to measure drug–plasma protein interactions: from high-throughput to in-depth analysis

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The selection of drug candidates with improved pharmacokinetics is essential to reduce the attrition rates during drug development and represents one of the big challenges faced by the pharmaceutical industry. Plasma protein binding (PPB) is an important parameter with significant implications for in vivo drug performance. Today, the most widely used techniques for PPB measurement in the pharmaceutical community are equilibrium dialysis (ED) and ultrafiltration (UF). However, these techniques have some limitations. Thus, we emphasize an alternative strategy, based on a global, new and easy-to-follow methodology, to screen and perform determination of PPB, using orthogonal techniques (i.e. liquid chromatography, capillary electrophoresis (CE), surface plasmon resonance (SPR) based biosensor). We anticipate that the increased knowledge gained through this strategy will lead to improved drug candidates.

Introduction

Drug research can be divided into two main phases: drug discovery and drug development. Drug discovery includes target identification and validation, hit and lead identification and finally lead optimization, whereas drug development comprises preclinical and clinical studies [1]. Drug research is a lengthy and costly process, taking an average of 15–20 years and US$1 billion to generate a successful medicine. However, this process is characterized by a high attrition rate, up to 90% by the end of the clinical trial process [2]. It is widely known that one reason for the lack of effectiveness of this process is poor ADMET properties [3]. The success of a drug is indeed determined not only by good efficacy but also by a suitable ADMET profile. Thus, an increasing effort has been made to improve understanding of the molecular characteristics that lead to potentially fatal liabilities in drug candidates and progress has been made in the prediction of human pharmacokinetics. In this context, plasma protein binding (PPB) is an important factor, along with lipophilicity and acid–base properties, that governs the *in vivo* distribution of drugs and their availability for pharmacologic activity [4,5].

When a drug binds to plasma proteins two drug fractions are obtained: the free drug fraction and the bound drug fraction. The free drug fraction is of the utmost importance for biologic effects because, in most cases, only this fraction can be distributed in the body, can elicit a pharmaceutical response and can be eliminated. The bound drug fraction serves as a drug reservoir for free-drug concentration and prolongs the duration of the drug action [6]. PPB can hence increase the pharmacokinetic half-life (by keeping the drug in the blood and restricting its clearance) but it can also restrict exposure to the therapeutic target (by reducing distribution into the tissues) [7,8]. The understanding and evaluation of this crucial parameter is required at several steps of drug research (Fig. 1).

Today, the most widely used techniques for protein binding measurement are equilibrium dialysis (ED) and ultrafiltration (UF). They are considered gold standards for measuring drug–protein interactions but suffer from several drawbacks, especially in terms of time delivery. This could be a reason why, although the strong impact PPB has on the pharmacokinetics of drugs has been well recognized for some time,

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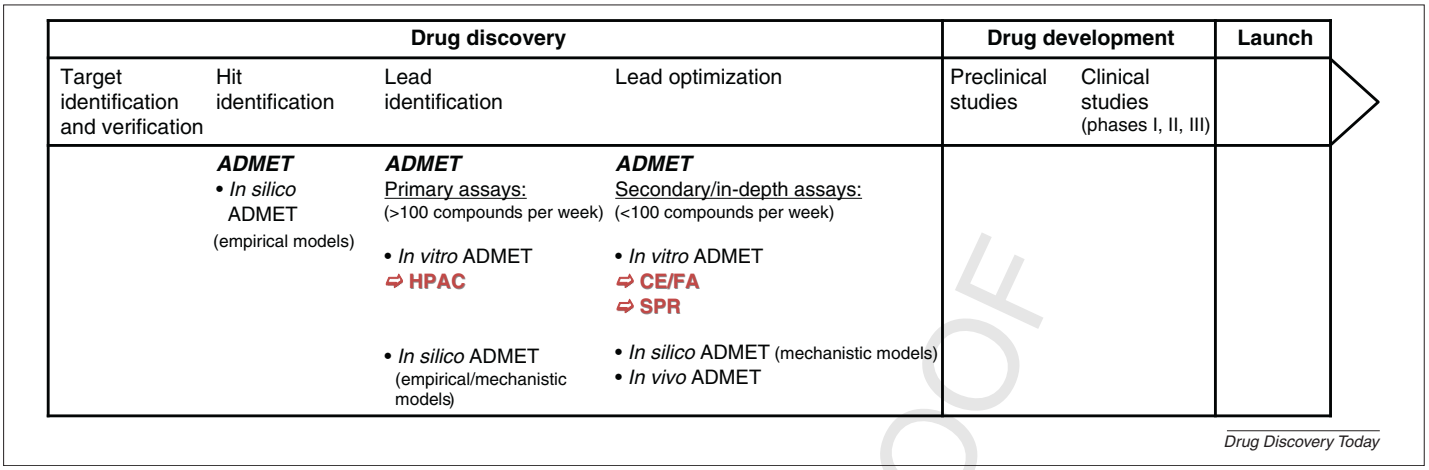

FIGURE 1

A schematic flow chart summarizing the process of drug research with an accent put on the AMDET properties that have to be evaluated at each step. In red are the methods that are proposed for *in vitro* characterization of plasma protein binding. *Abbreviations*: HPAC, high-performance affinity chromatography; CE/FA, capillary electrophoresis in frontal analysis mode; SPR, surface plasmon resonance based biosensor.

not enough attention has been paid to PPB during the whole drug research process. Given the importance of PPB to the *in vivo* drug performance, a complete and easy-to-follow methodology, anchored in the scheme of drug research, is proposed in this article.

Current practice

Different methods [9,10], such as ED, UF, ultracentrifugation (UC), spectroscopy, calorimetry, high-performance liquid chromatography (HPLC), capillary electrophoresis (CE) and surface plasmon resonance (SPR) based biosensors, are available to measure PPB but ED is considered the reference method.

ED is based on differences in molecular size and/or weight between drugs and proteins. In standard ED experiments two compartments are separated by a semipermeable membrane that acts as a molecular sieve to enable molecules smaller than a certain molecular size (i.e. drugs) to permeate through it, whereas large molecules, such as proteins, remain in the donor compartment. After a defined incubation time, equilibrium is reached and the free-drug fraction can be measured in the second compartment [11]. ED possesses the advantage in that analyses are performed in solution and that equilibrium is maintained during the whole experiment. However, equilibration times are long (typically 12–48 hours), an initial set of studies has to be performed to determine the time necessary for the system to reach equilibrium and an additional quantitation method is needed to determine the free fraction [12], such as HPLC–UV or HPLC–MS. Other pitfalls include: (i) drug instability under those long equilibration times; (ii) volume shift associated with the presence of proteins; (iii) nonspecific adsorption of compounds on the cell wall and dialysis membrane; and (iv) the Donnan effect. UF is another method commonly used in the pharmaceutical industry because it is based on the same principle as ED, except the analysis is sped up by the application of a pressure to force the solution through the membrane [13]. However, this method also shares the other drawbacks of ED. Both of these techniques obviously have some limitations, which narrow their application in drug research. We are convinced that an alternative strategy along with adapted analytical methods is needed to fulfill the requirements of the different stages of the drug discovery and development process.

Proposed strategy

The goal of the present strategy is to propose *in vitro* analytical methods to study drug–plasma protein interactions. *In silico* methods are also useful [14,15], especially in the early stages of the drug discovery process because they can handle a very large number of compounds but they are out of the scope of this paper. In the lead identification stage, when the number of new chemical entities (NCEs) is large, >100 compounds per week [2], a high-throughput method is required to classify compounds as weak, medium and strong protein binders. Later in the discovery process, in lead optimization when the number of compounds is lower (<100 compounds per week) in-depth analyses are required to gather more information on the binding process (Fig. 1) [2].

No defined cutoff values exist to identify strong binders but, according to the general trend found in the literature, we propose to define compounds with a binding percentage higher than 90% as strong binders. Although there are many components in blood that are capable of binding drugs, two proteins: human serum albumin (HSA) and α_1-acid glycoprotein (AGP) are known to bind a broad range of drugs with sufficient affinity to have a significant effect on drug action [16,17]. Knowing the protein involved in drug binding is interesting because a variation of the concentration of a specific protein, linked to a disease or genetic variation, can lead to a significant increase or decrease of the free, active drug fraction. That is the reason why the proposed strategy follows two flowcharts, one for HSA and one for AGP (Fig. 2).

For primary assays involving a large number of compounds, a method that provides fast measurements of a parameter characterizing the binding process is mandatory. High-performance affinity chromatography (HPAC) is well suited for HTS. The protein of interest is immobilized onto the chromatographic support. The retention time of an injected drug candidate gives indication on the strength of the interaction between this compound and the immobilized protein. The stronger the interaction the longer the compound will remain on the column. HSA and AGP columns are commercially available and it was demonstrated that these columns could be used to screen and classify compounds according to their affinity for both proteins [18,19]. Thus, the main goal of HPAC is to identify strongly bound compounds that are susceptible to induce drug safety issues or severe adverse effects (low clearance, low brain penetration, drug–drug interaction and loss of efficacy) [20]. Different strategies, such as the use of generic gradient conditions (buffer and

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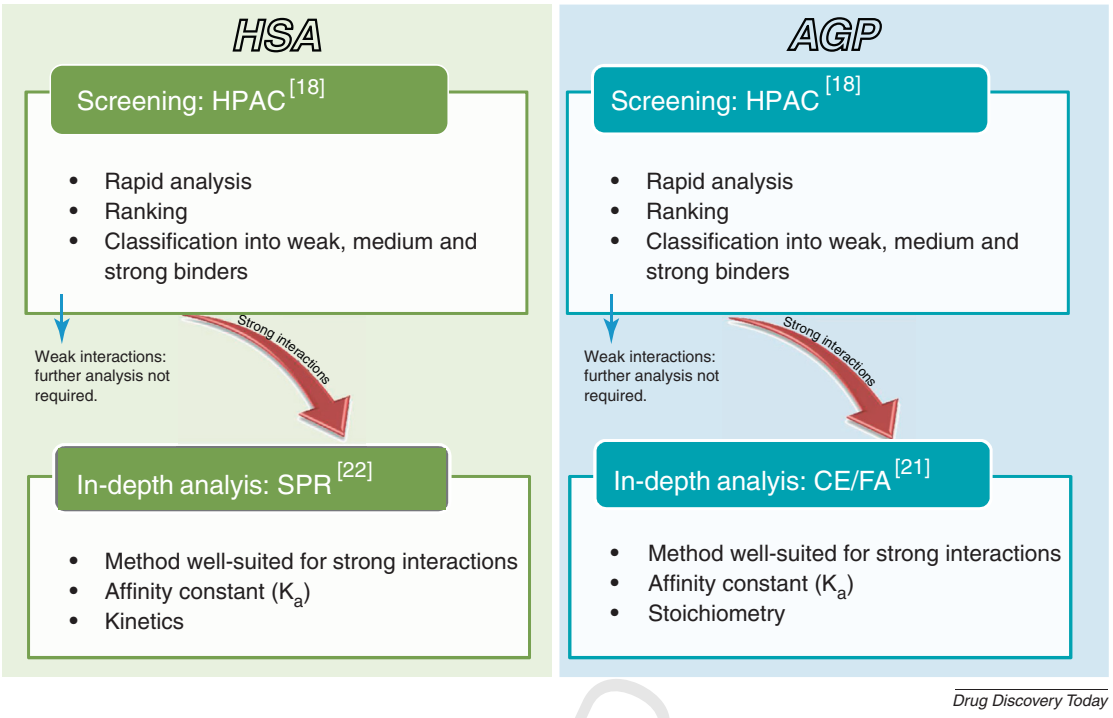

FIGURE 2

Proposed methodology to assess drug–plasma protein interactions. *Abbreviations*: HSA, human serum albumin; AGP, α1-acid glycoprotein; HPAC, high-performance affinity chromatography; SPR, surface plasmon resonance biosensor; CE/FA, capillary electrophoresis in frontal analysis mode; K_a, affinity constant.

organic solvent) and MS detection, are applied to offer HTS compatible with the large number of compounds. For example, when seven compounds are pooled in one sample less than 3 min per compound are required to obtain the approximate binding percentage [18]. HPAC is therefore a rapid and generic method used to discriminate highly bound compounds.

HPAC can also deal with low purity samples and the presence of degradation products is not a problem because HPAC is a separation method. Another advantage is its low sample consumption. Indeed, the same column packed with HSA or AGP can be used for about 500 injections and a single drug concentration as low as 5 μg/ml can be used for each drug. It has to be considered that the protein of interest is immobilized on a surface and not free in solution and that organic solvent is used to elute strongly bound compounds within a reasonable timeframe compatible with high-throughput analyses. This is however not an issue because it was demonstrated [18] that this method can classify compounds according to their affinity for HSA and AGP. The obtained information is certainly scarce but this result is enough and appropriate for a screening purpose. With this technique, the binding percentage range is limited to compounds bound at up to 99% (Table 1). For instance, naproxen and ibuprofen are too tightly bound to HSA to be eluted in the described conditions [18]. Hence, we propose to consider these compounds as very strong binders and to analyze them further with secondary assays adapted for stronger interactions (Fig. 2).

During the lead optimization step secondary assays are required for in-depth analysis of strong drug–plasma protein interactions. CE in frontal analysis mode (CE/FA) coupled with MS detection [21] and SPR [22] are useful orthogonal techniques for studying strong drug–protein interactions. CE/FA is a valuable technique because interactions are studied in solution. There is neither need for immobilization of the protein nor any labeling requirement. CE/FA can therefore be positioned as a new reference method for studying drug–plasma protein interactions. The principle of CE/FA is simple [23]: upon the injection of a mixture of drug–protein, a separation of the free and bound drug occurs. Careful method development is required to avoid disruption of the binding equilibrium during the analysis. It is ensured by the injection of larger sample volumes than with conventional CE, typically 20% of the capillary (~100 nl) instead of 1–2% (~10 nl), respectively. Once the maintenance of the equilibrium is assured, the free drug fraction can easily be determined. Several experiments at different drug:protein ratios must be performed to obtain the association constant and the stoichiometry of the interaction. With the use of MS detection sensitivity is increased by 20–40-fold compared with UV detection, and this enables easy characterization of very strong interactions (>99%). CE/FA-MS is well suited for the analysis of basic and neutral compounds, which are often involved in strong interactions with AGP. The analysis of acidic drugs, often involved in strong interactions with HSA, is more challenging because acidic compounds have to be ionized in negative ESI mode, which is less sensitive and less stable in terms of ESI-MS conditions than positive ESI (Table 1).

For strong drug–HSA interactions, SPR remains the method of choice. A SPR detector can be seen as a sensor of the change in refractive index that occurs at the surface anchoring the protein as complexes form or break during the binding reaction [24]. It was observed that the immobilization of HSA does not impact the obtained binding results, compared with results obtained in solution [22]. One of the major strengths of SPR is its ability to characterize strong to very strong drug–protein interactions (e.g. naproxen– and ibuprofen–HSA interactions) that give rise to a high response. Using SPR the same protein sample can be used for up to 500 injections, such as in HPAC experiments. Several drug:protein ratios are

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TABLE 1

Main features of HPAC, CE/FA, SPR and ED to assess drug–plasma protein interactions

	HPAC	CE/FA	SPR	ED
Information	Binding percentage	K_a, *n*	K_a, kinetics	K_a, *n*
Analysis time and/or compound throughput	<3 min +++	3 h ++	3 h ++	12–48 h +
Best suited for binding percentages	<99%	>99%	>99%	<99%
Usual volumes of samples	µl	nl	µl	µl
Usual order of concentration				
Drug	10–40 µM	1–5 µM	2–1000 µM	50–800 µM
Protein	One commercial column for 500 injections	10 µM	5 µg for 500 injections	25–150 µM
Cost	$$	$$	$$$	$
Main limitations	Use of organic solvent in the mobile phase Classification of compounds only	Analysis of acidic compounds difficult	Highly pure sample required Long method development	Long analysis time Highly pure sample required Nonspecific adsorption
Main advantages	Rapid	In-solution analysis Strong interactions	Strong to very strong interactions Kinetics	Gold standard In-solution analysis

Abbreviations: K_a, affinity constant; *n*, stoichiometry of the interaction; HPAC, high-performance affinity chromatography; CE/FA, capillary electrophoresis in frontal analysis mode; SPR, surface plasmon resonance based biosensor; ED, equilibrium dialysis.

required to obtain the binding information (i.e. the affinity constant). Another interesting aspect of SPR is its ability to provide real-time analysis, enabling qualitative and quantitative information on the kinetics of the interaction very easily [25]. By contrast, SPR requires highly pure samples because it is not a separation method and method development can be long and cumbersome (Table 1). However, once the immobilization protocol is validated SPR is easy to use, completely automated and, thus, well suited for routine analysis.

Concluding remarks

Because PPB has an important role for *in vivo* drug performance, *in vitro* experimental tools used to characterize drug–plasma protein interactions should be applied early in the drug discovery process to increase the success rate of drug research. The proposed methodology (Fig. 2) takes into account the requirements and specificity of the different stages of the drug research process. In a first step, HPAC [18] is proposed to screen compounds according to their affinity for HSA and AGP and identify strongly bound compounds. In a second step, strongly bound compounds should be analyzed with more-specific methods to generate more information. For AGP, CE/FA-MS [21] is well suited to characterize strong interactions because of the high sensitivity associated with MS detection. For drug–HSA interactions we recommend the use of SPR technology because it is simple, automated and easy to perform for high-affinity characterization. Furthermore, it has been demonstrated that binding properties of HSA are not perturbed when amine coupling is used to immobilize it on the sensor chip. Table 1 compares and summarizes the main features of each method.

Finally, because PPB controls the free, active drug concentration in plasma and in the compartments in steady-state equilibrium with plasma, we are convinced that PPB is a key attribute of a drug candidate that has to be taken into consideration along with other drug characteristics, such as lipophilicity, acido–basic properties, affinity for the target and selectivity. Moreover, comparisons based on the free, active drug fraction, using PPB adjusted potency, are important to help discriminate drug candidates.

Conflict of interest

The authors have no conflict of interest to declare.

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