The 'free drug hypothesis' : fact or fiction?

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Chapter 2

The 'free drug hypothesis' in pharmacodynamics: theoretical evaluation of the role of plasma protein binding.

Summary

Background Plasma protein binding (PPB) may affect both the pharmacokinetics (PK) and the pharmacodynamics (PD) of drugs. At present the theoretical basis of the influence of (alterations in) PPB on PK is well-established and is clearly defined as being either restrictive or non-restrictive. For the PD, however, plasma-protein binding (PPB) is considered to prevent the drug from binding to its physiological target ('free drug hypothesis') and this has sofar never been investigated in a systematic and quantitative manner. In theory, the PD of a drug is determined by the 'direct' competition between target and non-specific binding. In this respect both the affinity and the capacity of the target and PPB need to taken into consideration.
Objective The objective of this chapter was to develop a theoretical framework to explore the influence of non-specific PPB on the PD *in vivo*. To this end, PPB, target binding and the interaction between both were investigated in a step-wise manner on the basis of literature data and *in silico* simulation studies, taking into account the affinity as well as the capacity of the binding to the protein and to the target (receptor).
Approach Even though target site distribution is often required for a drug to evoke its pharmacological effect, a direct interaction between PPB and target binding was assumed as a first step. This assumption simplifies the complex influence of PPB on the cascade of events leading to drug effect.
Literature Receptor binding is typically characterised by a high affinity and a low capacity, while for PPB the opposite is the case. Albumin is considered the main binding protein because of its high concentration in plasma (600 μM) and drug binding to this protein is generally non-saturable (linear with drug concentration). Binding to the other major binding protein in plasma, alpha-1-acid glycoprotein (AGP), is saturable (drug concentration dependent) under normal physiological conditions. AGP concentrations, however, are known to increase under pathophysiological conditions (9 μM to 72 μM) and the simulations show that under these conditions the binding is no longer saturable. Although the concentration and the binding capacity of AGP are smaller than of albumin, the drug-binding affinity is in general higher which contributes to the significance of binding to AGP.
Simulations The results show that non-restrictive PPB for drugs is possible because of the saturable nature of the receptor binding. Non-restrictive binding, however, requires a large difference in the affinity for protein and receptor (>1000-fold). In case of a very large difference in the affinity for protein and receptor, a change in PPB will not result in a shift in the total plasma concentration-effect relationship. *Conclusions* In conclusion, PPB will in general be restrictive to PD for small molecules which display a rapid equilibrium for both receptor- and protein binding. Only if the difference in affinity is more than 1000-fold, an alteration in PPB will not result in a shift in the concentration-effect relationships of these drugs.

2.1 Plasma protein binding (PPB)

Plasma proteins

In blood, drugs bind to serum albumin, α1-acid glycoprotein (AGP) (Kremer *et al*., 1988), and other blood constituent like lipoproteins, erythrocytes and α-, β-, γ-globulins (Wright *et al*., 1996). The main binding proteins for drugs in plasma are albumin and AGP (Israili and Dayton, 2002; Bertucci and Domenici, 2002).

Albumin is the most important drug-binding protein *in vivo* due to its high concentration in plasma. Binding to albumin normally accounts for 50-60% of the total plasma protein, with concentrations in humans of 35-

50 g/L (~500-700 μM) (Paxton, 1985; Doweiko and Nompleggi, 1991; Wright *et al.*, 1996) and slightly less in rats (Murai-Kushiya *et al.*, 1993a). In addition to being a carrier protein for a number of endogenous (e.g. fatty acids and bilirubin) and exogenous compounds, the main physiological functions of albumin are the maintenance of colloid osmotic pressure and of a stable blood pH (Doweiko and Nompleggi, 1991). Albumin is synthesised in the liver and as a consequence its concentrations may reduce in liver diseases (e.g. cirrhosis). Albumin has several high- and low-affinity binding sites and is mainly involved in the binding of acidic drugs (Piafsky, 1980; Notarianni, 1990; Day and Myszka, 2003).
AGP is mainly involved in the binding of neutral and basic drugs (Kremer *et al.*, 1988; Murai-Kushiya *et al.*, 1993b; Kopecky, Jr. *et al.*, 2003). In addition to being a carrier protein, AGP is considered an anti-inflammatory and immunomodulatory agent, playing a role in inflammation and various pathophysiological conditions (Kremer *et al.*, 1988; Fournier *et al.*, 2000). It is known that infection, inflammation or severe injury induces a cascade of reactions called the acute phase response, in which plasma concentrations of AGP increase about ten-fold (Kushner, 1982; Murai-Kushiya *et al.*, 1993a; Fournier *et al.*, 2000).

Measurement of PPB
Methods for determination of drug-protein binding in plasma include microdialysis, equilibrium dialysis, ultrafiltration, dynamic analysis, ultracentrifugation, gel filtration, electrophoresis, spectrophotometry and enzyme kinetic methods (Pacifici and Viani, 1992; Wright *et al.*, 1996). Though methods are abundant for determination of PPB, most methods are rarely used. The most commonly used methods are microdialysis, equilibrium dialysis and ultrafiltration (Wright *et al.*, 1996). Since PPB is considered a key property for drugs, it is determined frequently at non-saturating conditions and typically reported as the percentage bound or the free fraction (Pacifici and Viani, 1992; Talbert *et al.*, 2002).
For drugs with extremely high PPB (>99%), the accurate and precise determination of the 'free fraction' is difficult with the presently used techniques. New methods for determining PPB are, thus, required. Recently new techniques like frontal chromatography and biosensor assays have become available for the rapid screening of the interaction between drug and protein (Ostergaard and Heegaard, 2003; Schuhmacher *et al.*, 2004; Torreri *et al.*, 2005). More specifically, the affinity of a drug for plasma proteins (*Kdp)* can be quantified reasonably easy.

2.2 PPB in pharmacokinetics

Pharmacokinetics (PK) is the study of the fate of a drug in the body and is typically divided into absorption, distribution, metabolism and excretion (ADME). The influence of (alterations in) PPB on the major PK parameters is well-established (Wilkinson, 1983). The absorption rate of a drug is mainly dependent on the concentration in the gastrointestinal tract and is, as a result, not affected by PPB. The bioavailability, though, is affected by PPB, since the free concentration is one of the determinants of the first pass effect. The maximum oral bioavailability based on the well-stirred model is defined by equation 1 (Rowland and Tozer, 1995).

$$F = 1 - E_H = \frac{Q_H}{Q_H + fu_b \cdot CL_{\text{int}}} \qquad (1)$$

where, F is the maximal bioavailability, E_H is the hepatic extraction ratio, Q_H is the hepatic blood flow, fu is the free fraction in blood and CL_{int} is the intrinsic clearance.
The volume of distribution (V) is the primary PK parameter which describes the distribution of a drug and

may be defined by equation 2 (Rowland and Tozer, 1995).

$$V = V_P + V_T \cdot \frac{fu}{fu_T} \quad (2)$$

where V_P and V_T are the volume of plasma and tissue (aqueous volume outside plasma), and *fu* and fu_T are the unbound fractions in plasma and tissue, respectively. Theoretically, the volume of distribution will increase with an increase of the free fraction (reduction of PPB). Generally speaking, for highly protein bound drugs, often lipophilic molecules, the volume of distribution will increase upon a decrease in the extent of PPB. For hydrophilic drugs, the PPB is often low, and changes do not change the volume of distribution significantly. The distribution of these drugs into tissues is limited and as a consequence the volume of distribution is almost equal to the extracellular volume of the body water (~14L in adults).
The clearance (*CL*) of a drug can be restrictive or non-restrictive with regard to PPB (Rowland and Tozer, 1995). The clearance is considered restrictive if only the unbound drug is available for clearance from the system. Conversely, non-restrictive clearance is defined as clearance which is independent on the free drug. Based on the well-stirred model, the hepatic clearance can be defined by equation 3 (Pang and Rowland, 1977).

$$CL_{b,H} = Q_H \cdot E_H = \frac{Q_H \cdot fu_b \cdot CL_{\text{int}}}{Q_H + fu_b.CL_{\text{int}}} \quad (3)$$

where Q_H is the hepatic blood flow; E_H is the hepatic extraction ratio; CL_{int} is the intrinsic clearance and *fu* is the fraction unbound in blood. Clearance is nearly independent of the free fraction for high extraction ratio drugs (E_H > 0.7, e.g. propranolol). The clearance for these drugs is perfusion rate-limited and approaches the blood flow of the eliminating organ (CL_H » Q_H). Conversely, the clearance for low extraction ratio drugs (E_H < 0.3, e.g. diazepam) is highly dependent on the free fraction (CL_H » $fu.CL_{int}$). The influence of *fu* on the renal clearance of drugs is comparable to those described for hepatic clearance. As a result of the influence of PPB on clearance (*CL*) and volume of distribution (*V*), the secondary PK parameters (e.g. half-life) are affected by *fu* as well. The overall influence of PPB on the PK of a drug is, thus, the result of the sum of the individual PK parameters (Table 1) (Mehvar, 2005).

Table 1, Influence of alteration in PPB on individual PK parameters and the consequential changes in the total and free drug concentration in plasma (partially adapted from Mehvar 2005)

Drug		Parameters and Variables					
CL	***V***	***fu***	***V***	***CL***	***t½***	AUC_{total}^{A}	AUC_{free}^{A}
High	High	↑	↑	↔	↑	↔	↑
		↓	↓	↔	↓	↔	↓
Low	Low	↑	↔	↑	↓	↓	↔
		↓	↔	↓	↑	↑	↔
Low	High	↑	↑	↑	↔	↓	↔
		↓	↓	↓	↔	↑	↔
High	Low	↑	↔	↔	↔	↔	↑
		↓	↔	↔	↔	↔	↓

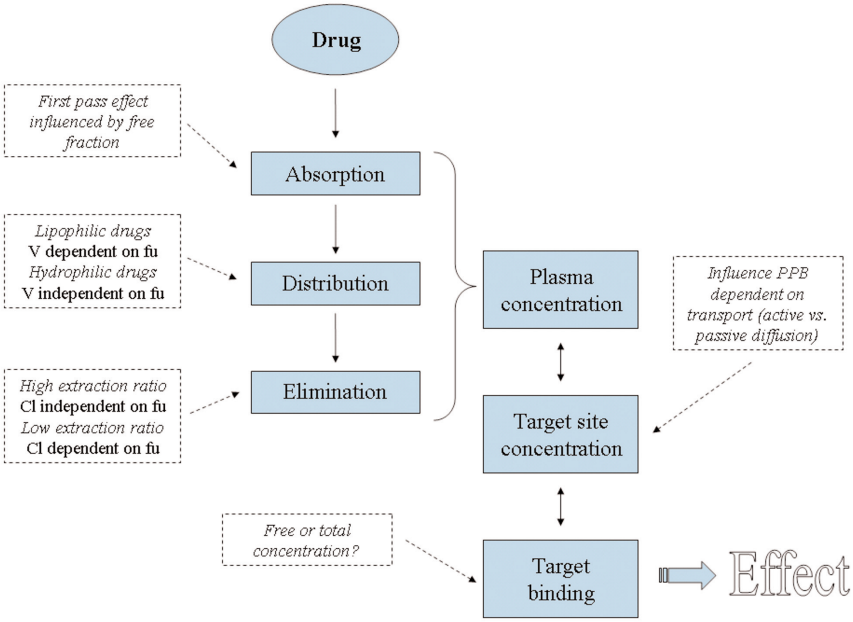

Figure 1, Overview of the influence of PPB on drug action in vivo

2.3 PPB in pharmacodynamics

The pharmacological effect of a drug is dependent on both the pharmacokinetics (PK) and the pharmacodynamics (PD). The PK determines the exposure of the target site to the drug in terms of the concentration *versus* time profile. On the other hand, the PD, describes the processes leading to the pharmacological effect including the binding of the drug to its physiological target (e.g. receptor, transporter, enzyme), the activation of the target and the transduction processes leading to the effect of the drug (e.g. heart rate). As a consequence, the influence of PPB on drug action *in vivo* is complex, since PPB affects multiple parts of the cascade leading to drug effect (figure 1).

The 'free drug hypothesis' states that the pharmacological activity of a drug is correlated with the unbound rather than the total concentration in plasma (Israili, 1979). The hypothesis is based on the assumption that the binding to plasma proteins may restrict the distribution to the target site and the binding to the physiological target in the body.

There is some disagreement in the literature on the relevance of PPB for the clinical efficacy of drugs. Traditionally it has been felt that protein binding can limit target exposure. However, an examination of the relationship between the percentage of PPB and observed effects show that the relationship is not that simple.

For certain drugs like benzodiazepines, opiates, and steroids experimental data indicate that it is indeed the free concentration that determines the intensity of the response (Derendorf *et al.*, 1993; Mandema *et al.*, 1991; Cox *et al.*, 1998; Visser *et al.*, 2003). A number of reviews have addressed the clinical importance of drug protein binding (Sellers, 1979; Sparreboom *et al.*, 2001; Benet and Hoener, 2002). Interestingly, the overall conclusion is that PPB of drugs and possible drug displacement interactions have little to no clinical significance. Benet concluded that for a large number of clinically used drugs (25 of 456) changes in PPB are not significant, since the AUC_{free} does not change with alterations in the free

fraction in plasma (Benet and Hoener, 2002). This conclusion, however, is based on the general consensus that the pharmacological effect is related to exposure to unbound drug concentration ('free drug hypothesis') and as such does not assess the possible influence of PPB on PD.
Interestingly, for other drugs (e.g. A_1 adenosine agonists, selective CCK1 receptor antagonists and corticosteroids) available evidence seems to indicate that the total rather than the free concentration determines the response (Wald *et al*., 1992; Van Der Graaf *et al*., 1997; Gerskowitch *et al*., 2007). The exact mechanisms which cause the invalidity of the 'free drug hypothesis' in these studies are unknown; however, some possible explanations were described. Wald described that corticosteroids bound to transcortin were shown to cross vesicular membranes unimpeded (Wald *et al*., 1992). On the other hand, Gerskowitch hypothesized that the distribution processes are possibly fast compared to the off rate from the receptor Gerskowitch *et al*., 2007. Some studies have indeed shown that the free concentration in plasma is not always the best predictor of the free concentration in brain because of for example efflux transporters and non-linear protein binding (Hammarlund et al, 1997; Marchand *et al*., 2000; Liu *et al*., 2006; Groenendaal et al, 2007). The influence of PPB on target site distribution is, thus, complicated especially in the case of active transport from or to the target site. Finally, PPB may directly affect drug-target binding due to an interaction of the protein with the target (Sattari *et al*., 2003; Rodriguez *et al*., 2004). In this respect, Rodriguez et al., have shown the benefit of integrated PKPD studies for complex interactions such that of AGP with the methadone PK and PD. In addition, Proost et al., described a quantitative analysis on the influence of PPB, tissue binding and receptor binding on the potency and on the time course of action of drugs using an extended PKPD model (Proost *et al*., 1996). In that publication the impact of changes in both PK and PD parameters was assessed under the assumption that free drug is distributed to the tissue and binds to the target. To our opinion a systems approach is essential in investigations concerning the influence of PPB on PD because of the complexity of the problem.

The 'free drug hypothesis' in drug discovery and development
In drug discovery, high PPB (>95%) is considered to prevent the drug from binding to its physiological target (i.e. receptor or enzyme) and is, therefore, considered a non-favourable property for a new chemical entity (NCE). This has led to the practice of using PPB as a selection criterion for new drug candidates (Trainor, 2007). The (experimental) evidence for the 'free drug hypothesis' in PD, however, remains questionable and is, therefore, subject to debate. The objective of this chapter is to develop a theoretical framework to explore the influence of non-specific PPB on the PD *in vivo*. To this end, PPB, target binding and the interaction between both were investigated in a step-wise manner on the basis of literature data and *in silico* simulation studies, taking into account affinity as well as capacity for the protein and for the target (receptor).

2.4 Key determinants of the impact of PPB on PD

Protein binding affinity
Affinities (equilibrium dissociation constants) for protein binding are not commonly reported for drugs. In general, PPB of a drug is determined under non-saturating conditions and characterised as the percentage bound and/or the free fraction. Individual values of the binding affinity (*Kdp*) to albumin or AGP are only determined in special cases. Some of the extreme values for affinities for the plasma proteins albumin and AGP reported in literature are shown in table 2.
Since pertinent information on the binding affinity of drugs plasma proteins is not readily available, in the

Table 2, Examples of drugs with high and low affinity binding and respective affinity (Kdp) for albumin and AGP.

Protein	Drug/Ligand	Affinity	Binding (%)	*Kdp* (M)	Reference
AGP	*Clindamycin*	High	~80	9.4×10^{-10}	(Kays *et al.*, 1992) (Son *et al.*, 1998)
AGP	*Phenobarbital*	Low	-	8×10^{-2}	(Schley and Muller-Oerlinghausen, 1983)
Albumin	*Dicumarol*	High	>99.9	1.9×10^{-7}	(Day and Myszka, 2003)
Albumin	*Quinine*	Low	22	2.5×10^{-3}	(Day and Myszka, 2003)

present investigation this information was derived indirectly from estimates of the free fraction under non-saturating conditions. The relationship between the free fraction (φ) and the affinity constant for protein binding (*Kdp*) under the condition of linear protein binding (e.g. albumin) is described by equation (4).

$$\varphi = \frac{1}{\frac{[P]}{Kdp} + 1} \tag{4}$$

in which φ is the free fraction, *Kdp* is the equilibrium dissociation constant (affinity) of the drug and *[P]* is the protein concentration (appendix A).

The theoretical relationship between the free fraction (φ) and the affinity constant for protein binding (*Kdp*) in absence of receptor and under the condition of non-linear protein binding (e.g. AGP) can be described by equation (5).

$$\varphi = \frac{[D] - [P] - Kdp + \sqrt{([D] - [P] - Kdp)^2 + 4 \cdot Kdp \cdot [D]}}{2 \cdot [D]} \tag{5}$$

in which φ is the free fraction, *Kdp* is the equilibrium dissociation constant (affinity) of the drug, *[D]* is the drug concentration and *[P]* is the protein concentration (appendix A). On basis of the extreme values reported in table 1 and the equations for protein binding the affinity range for drug-protein binding was constructed. The affinity range for both albumin and AGP binding is shown in addition to the range for receptor binding in figure 2. The range for AGP binding was found to be larger than for albumin, which is most probably due to the more specific binding to AGP. Comparison of affinity values on individual compound basis showed that in general, binding to plasma proteins is less tight than the binding of the drug to the target, also on individual basis.

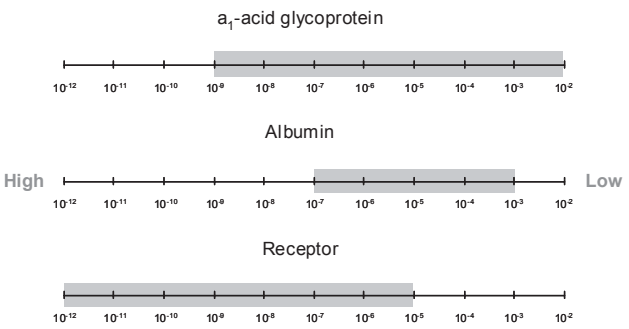

Figure 2, Range in equilibrium dissociation constants (Kd (M)) for drug binding to both protein (non-specific) and receptor (specific). The Kd represents the affinity (equilibrium dissociation constant) of the drug for protein (Kdp) and receptor (Kdr) and the values were obtained from literature.

Target binding affinity

The pharmacological effect of a drug is brought about by binding to its physiological target. In our approach we have taken the receptor as one of the most important targets. The receptor equilibrium dissociation constant (*Kdr*), is a measure of the receptor-affinity. As a consequence pertinent information on the binding affinities of drugs for their pertinent receptor is readily available in the literature and has also been summarised in standard texts such the BJP Guide to Receptors and Channels (Alexander *et al*., 2008). The range in *Kdr* for receptor binding was assessed searching literature for low- and high-affinity drugs instead of providing a complete overview of receptor binding affinities.

A wide range of receptor binding affinities of drugs has been reported with values ranging from the millimolars to the picomolars. Typically binding is considered to be of low affinity if *Kdr* of the drug for a given receptor lies between mM and μM values. Low receptor binding affinity drugs, which are typically used clinically, usually have a receptor binding affinity of μM. Presumably because drugs with an even lower affinity (>μM) would either be unselective or ineffective and would, thus, require a high dose to be effective or cause safety issues. Examples of low receptor binding affinity drugs which are used clinically are rare. Tramadol is considered a low affinity drug (*Kdr* = 2.4 μM), but it should be noted that it has a metabolite which is formed *in vivo* and which displays a much higher affinity for the target than the parent compound (*Kdr* = 3.4 nM) (Gillen *et al*., 2000). Other illustrations of low affinity drugs are NSAID's, like acetaminophen and ibuprofen (Warner *et al*., 1999). Compounds with a *Kdr* value between μM and nM are regarded as medium affinity drugs (e.g. metoprolol *Kdr* = 44.6 nM) (Abrahamsson *et al*., 1988).

For high receptor affinity binding, the equilibrium dissociation constant is normally in the range nM to pM. Drugs are considered to have a high receptor binding affinity if *Kdr* is in the range of nM. Quite a number of small molecular weight drugs display a high affinity for its target (e.g. paroxetine *Kdr* ~ 0.1 nM) (Owens *et al*., 1997).

Extremely high receptor binding affinity (< pM) is exceptional for small molecules and is a property which is mainly observed for radioligands, which have been specifically designed for radioligand binding studies, and for antibodies/therapeutic proteins (e.g. [^{3}H]-CGP 12,177 *Kdr* = 40 pM, mAB hVEGF *Kdr* = 8 pM) (Schlaeppi *et al*., 1999; Niclauss *et al*., 2006;). The constructed affinity range for drug-receptor binding is, as a result, 1 pM (10^{-12} M) to 10 μM (10^{-5} M) and is shown in figure 2.

Target and protein binding capacity

Drug binding to plasma proteins is typically with low affinity (non-specific) and high capacity binding. Non-linear protein binding is an exception and observed only under special conditions like, high drug concentrations (e.g. antimicrobials like cefazolin) and binding to very specific proteins (i.e. retinol binding to retinol binding protein (RBP)) (Vella-Brincat *et al*., 2007; Noy and Xu, 1990). In addition, for the two most important drug-binding proteins, albumin and AGP, the total amount of plasma protein in the body largely exceeds the total amount of targets like enzyme or receptor. Protein binding is, therefore, in most cases non-saturable (drug concentration independent or linear). Saturable albumin binding at therapeutical drug concentrations is only reported for a small number of drugs. Due to its high concentration *in vivo*, the binding to albumin is seldom saturable and is, therefore, only observed for drugs which are administered at high doses (e.g. the anticancer agent Indisulam) (Zandvliet *et al*., 2006). Saturable AGP binding, on the other hand, is more frequently observed (e.g. isradipine, disopyramide and bepridil) (Pritchard *et al*., 1985; Siddoway and Woosley, 1986; Pinquier *et al*., 1989). The overall PPB for some drugs (e.g. *l*-propranolol) may become non-linear due to a combination of saturable and non-saturable binding to AGP and Albumin, respectively (Brynne *et al*., 1998).

We have chosen an *in silico* approach to assess the influence of PPB on drug effects and focussed on small molecule drugs, since therapeutic proteins usually do not bind to albumin or AGP. In addition, a single binding site was assumed for both receptor and protein binding. This assumption simplifies the equations used in the simulations. For receptor and AGP, this assumption is generally valid. On the other hand, albumin is known to have multiple low and high affinity binding sites for a number of drugs and this might enlarge the capacity of the protein for the drug (capacity = number of binding sites multiplied by the concentration protein). The binding of the drug to albumin was considered to be independent of the drug concentration (linear) and drug concentration dependent (non-linear) for AGP. In addition, the approach included the assessment of the influence of a change in the AGP concentration, due to pathophysiological conditions.

In this chapter we present simulations on the influence of PPB on the receptor binding of drugs. In these simulations the values of the protein concentration in plasma were obtained from the literature (Rowland and Tozer, 1995). Moreover in the case of non-saturable protein binding the protein concentration was assumed to be equal to the plasma albumin concentration (*[P]* =550 μM).

For saturable protein binding, the free fraction is not only a function of the affinity of the drug for the protein, but also dependent on the concentration of the drug (equation 5). In the simulations of saturable protein binding, the protein concentration was assumed to be equal to the plasma AGP concentration under normal conditions (*[P]* =9 μM). In figure 3, the free fraction was simulated for several drug concentration (0.5-50 μM) using the drug-protein affinity (*Kdp*). The results showed that the free fraction increased (0.01 – 0.82) with increasing drug concentrations for compounds with a *Kdp* smaller than 10^2 μM. Saturable protein binding is, thus, expected for AGP under normal conditions, since therapeutic drug concentrations typically range between 0.5 and 50 μM.

AGP concentrations increase under pathophysiological conditions (~72 μM). Under conditions of elevated AGP levels protein binding is considerably less saturable for drug concentrations varying between 0.5 and 50 μM (Figure 4). Slight saturable AGP binding is only observed for drugs with a *Kdp* value of approximately 10 μM. In addition, with very small Kdp (<1 μM) values, the free fraction is slightly dependent on the drug concentration. For a Kdp of 1 μM, the free fraction changes from 0.04 to 0.01 with a drug concentration from 50 to 0.5 μM. As expected, the relationship between *Kdp* and *φ* approached the relationship obtained for non-saturable albumin binding (equation 4; results not shown).

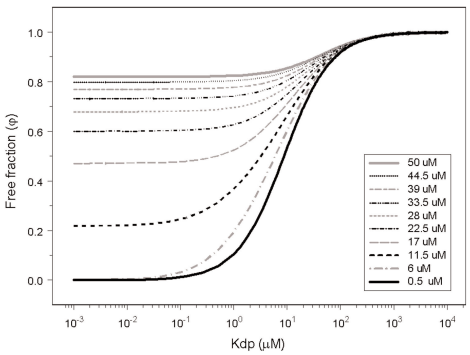

Figure 3, Simulation of the free fraction (φ) on basis of the affinity for protein, Kdp (μM). A protein concentration of 9 μM (normal AGP concentration) and several drug concentrations (0.5-50 μM).

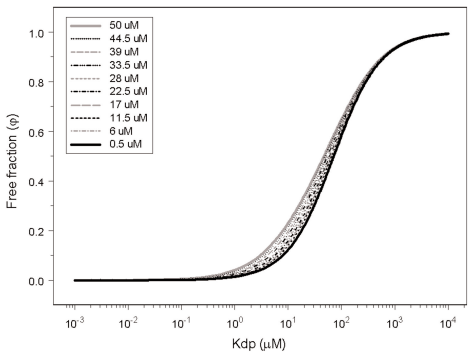

Figure 4, Simulation of the free fraction (φ) on basis of the affinity for protein, Kdp (μM) and a protein concentration of 72 μM (Acute phase reaction AGP concentration).

The amount of receptor present in the system varies between species, but also between tissues. Moreover, receptor concentrations *in vivo* vary greatly between receptor classes. A common property of the amount of receptor is that it is small compared to the amount of plasma proteins in a certain system. For the β1-adrenoceptor in rat heart the receptor concentration is 0.001 μM and the difference between receptor and protein concentration is, hence, more than thousand fold (Juberg *et al*., 1985). As a consequence receptor binding is considered highly saturable and, accordingly, non-linear when compared to PPB. Target-mediated disposition is typically clearly observable in the PK of therapeutic proteins, but for small molecule drugs, however, the PK is typically not affected by a very high-affinity for the receptor, because of the small receptor capacity.

2.5 The interaction between receptor binding and protein binding

The pharmacological effect of a drug is the result of the competing interactions between receptor binding and non-specific binding in the body. It is therefore important to understand the influence of PPB on the receptor occupancy of the drug as a direct determinant of the drug effect intensity. A schematic representation of the competing interaction between target binding and protein binding is displayed in figure 5.

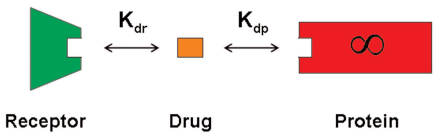

Figure 5, Schematic representation of drug binding to a receptor in the presence of plasma protein

Theoretically, the free drug concentration *[A]* in the case of binding to a receptor in the presence of protein can be described using equation 6. A limited (saturable) and unlimited (non-saturable) reservoir was assumed for receptor and protein, respectively.

$$[A] = \frac{[D_T] - [Rt] - Kdr - \frac{[P]}{Kdp} \cdot Kdr + \sqrt{([D_T] - [Rt] - Kdr - \frac{[P]}{Kdp} \cdot Kdr)^2 + 4 \cdot Kdr \cdot (1 + \frac{[P]}{Kdp}) \cdot [D_T]}}{2 \cdot (1 + \frac{[P]}{Kdp})} \quad (6)$$

in which *[Rt]* is the total receptor concentration, *Kdr* is the equilibrium dissociation constant (affinity) of the drug for the receptor, *[D_T]* is the drug concentration, *[P]* the protein concentration (*[P]*) and *Kdp* the equilibrium dissociation constant (affinity) of the drug for the protein (appendix A). Subsequently the percentage receptor occupancy (RO) (i.e. the concentration of occupied receptors divided by the total receptor concentration) can be calculated on basis of the free drug concentration (*[A]*), the total drug concentration (*[D_T]*), the protein concentration (*[P]*), the affinity of the drug for the protein (*Kdp*) and the total receptor concentration (*[Rt]*) using equation 7.

$$RO = \frac{[RA]}{[Rt]} * 100 = \frac{[D_T] - [A] - \frac{[P]}{Kdp} \cdot [A]}{[Rt]} * 100 \quad (7)$$

For most drugs PPB is non-saturable and, therefore, the protein concentration was assumed to be equal to the albumin concentration (*[P]* =550 μM) in plasma. In order to allow a comparison between the theoretical and the experimental work presented in this thesis (see chapter 2) the receptor concentration

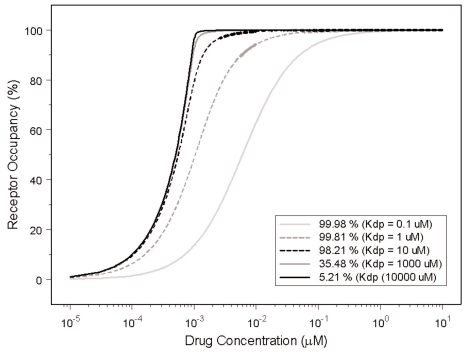

Figure 6, Influence of PPB on receptor occupancy (%) for albumin binding (550 μM) for a drug with high affinity for the receptor (Kdr = 1e-6 μM)

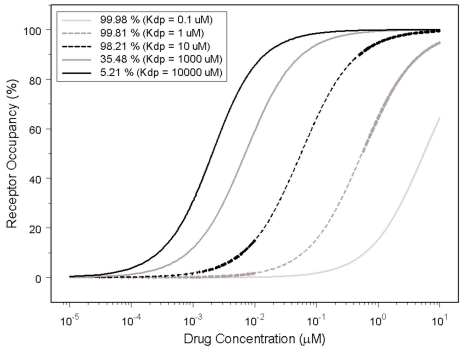

Figure 7, Influence of PPB on receptor occupancy (%) for albumin binding (550 μM) for a drug with medium affinity for the receptor (Kdr = 1e-3 μM)

was assumed to be equal to the concentration of β1-adrenoceptor in rat heart (*[Rt]* =0.001 μM) (Juberg *et al*., 1985). Finally in the simulations the drug concentrations ranged from 1.10^{-5} to 1.10^{1} μM.

The influence of PPB on the target occupancy was assessed by simulating receptor occupancies under varying PPB percentages. The percentages were calculated using equation 4 and the range of the values used was obtained from figure 2. Figure 6 displays the relationship between the total plasma concentration and the receptor occupancy for a drug with a high receptor affinity (*Kdr* = 1e-6 μM) under varying PPB.

For these high affinity drugs, the total plasma concentration *versus* receptor occupancy curve is almost identical for compounds with a protein binding ranging between 5% (*Kdp* = 1.10^{-2} M) and 98% (*Kdp* = 1.10^{-5} M). Thus in these simulations the binding affinity to the receptor in these simulations is 1.10^{5} to 1.10^{10} times higher compared to the affinity to the binding protein. Under these conditions the % RO and thereby presumably also the pharmacological effect intensity is non-restrictive with regard to PPB. PPB is defined as non-restrictive if the shift in receptor-occupancy is not directly related to the difference in PPB. Only at very high PPB at values exceeding 99% a shift in the plasma concentration *versus* target occupancy relationship towards higher plasma concentrations is observed. For drugs with a very high receptor affinity compared to the protein affinity, the 'free drug hypothesis' may as a consequence be invalid since the non-restrictive character of PPB.

A quantitatively different situation is observed for a compound with an intermediate target affinity as is shown in Figure 7. Here significant shifts in the plasma concentration *versus* % RO relationships are observed at PPB values exceeding 35% (*Kdp* = 1.10^{-3} M), indicating that PPB is restrictive with regard to the target occupancy. PPB is defined as restrictive for the PD if the shift in the receptor-occupancy curve is proportional to the difference in PPB. The simulations for a drug with a low receptor affinity showed that at the used drug concentrations % RO was low and high drug concentrations were needed to obtain receptor occupancy at all (results not shown).

The AGP concentration is known to increase during an acute phase reaction. To assess the influence of such a change on the PD of a drug, the receptor occupancy curve was simulated for four different conditions, namely (A) high affinity for receptor and protein, (B) low affinity for receptor and protein, (C) high receptor and low protein affinity and finally (D) low receptor and high protein affinity (figure 8).

The results showed that with increasing AGP concentration a significant shift in % RO is only observed in the case of high affinity for both receptor (*Kdr* = 1.10^{-6} μM) and protein (*Kdp* = 0.001 μM). It can,

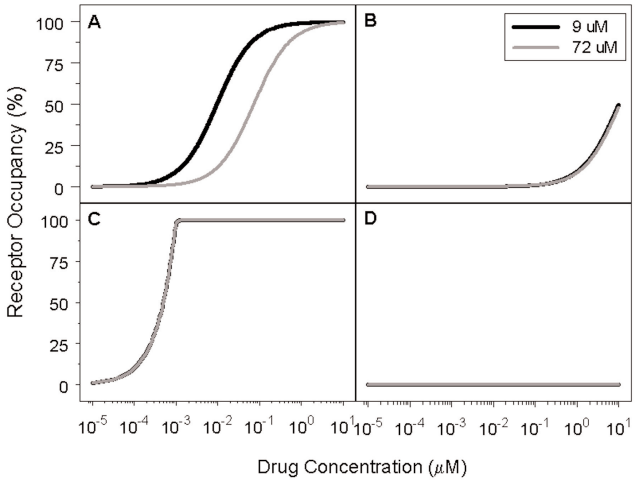

*Figure 8, Receptor Occupancy (%) plotted against drug concentration for four situations: **A.** High receptor (Kdr = 1e-6 μM) and high protein affinity (Kdp = 0.001 μM); **B.** Low receptor (Kdr = 10 μM) and low protein affinity (Kdp = 1000 μM); **C.** High receptor (Kdr = 1e-6 μM) and low protein affinity (Kdp = 1000 μM); **D.** Low receptor (Kdr = 10 μM) and high protein affinity (Kdp = 0.001 μM). Receptor occupancy is simulated for a protein concentration of 9 μM (normal AGP concentration) and 72 μM (AGP concentration during an acute phase reaction)*

therefore, be concluded that in case of a drug with high receptor as well as high protein binding affinity, a shift in receptor occupancy is to be expected upon changes in protein binding capacity. The 'free drug hypothesis' is thus valid for these compounds.

Finally, the influence of the difference in binding affinity for receptor (*Kdr*) and protein (*Kdp*) on % RO was assessed (figure 9). The % RO was simulated for a single drug concentration of 5 μM and plotted against the plasma-receptor affinity ratio (r_{PR}). r_{PR} is defined as the value of *Kdp* divided by the value of *Kdr*. In this manner, a high value of r_{PR} indicates a high receptor affinity compared to the protein affinity. The r_{PR} was obtained using the extreme values for binding affinity obtained from literature and ranged between 1.10^{-4} and 1.10^{7}. Receptor occupancy increased with increasing values for r_{PR} thus the higher the value for *Kdp* relative to *Kdr*, the higher the receptor occupancy.

At r_{PR} values smaller than 10, the percentage receptor binding is negligible (<10%). A steep increase in the percentage receptor occupancy is observed for r_{PR} values between 10 and 1000. Finally, at values of r_{PR} larger than 1000 the receptor is fully occupied (>90%).

Under the assumption that drug effect is directly related to the receptor occupancy, the performed simulations showed that non-restrictive protein binding (RO>90%) with regard to the PD is only observed

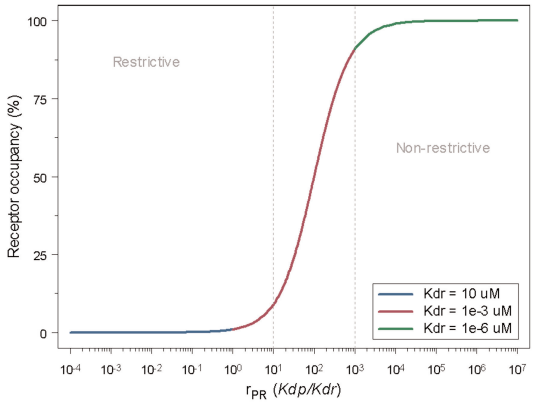

Figure 9, Receptor occupancy plotted against the ratio (r_{PR}) between affinity for protein and affinity for receptor. Restrictive (r_{PR}<10) and non-restrictive (r_{PR}>1000) protein binding is indicated in the graph.

for drugs with a very high affinity for the receptor compared to the affinity for the protein (<1000-fold difference).

2.6 Conclusions

An *in silico* approach has the advantage that multiple hypothesis can be tested in a reasonably straightforward manner without the need of extensive experiments. In addition, an *in silico* approach can provide prior information with regard to the design of crucial experimental studies. In addition, the outcomes of simulations can be used for the interpretation of experimental data obtained *in vitro* or *in vivo*. An *in silico* approach was chosen to assess the characteristics of PPB and the influence of PPB on receptor occupancy. Meaningful parameter estimates for the simulations were obtained from literature to provide insight in the "direct" competition between drug-protein and drug-receptor binding.

The simulations presented in this chapter show that the free fraction of a drug in plasma is influenced by both the affinity and the capacity of the binding at plasma proteins. At relevant drug concentrations the PPB is usually non-saturable. Specifically, binding at albumin is in almost all cases independent of drug concentration while AGP binding is only expected to be dependent on drug concentration at normal physiological concentrations of AGP.

The percentage binding is dependent on the affinity of the drug for the protein, the concentration of plasma proteins and the number of binding sites (Paxton, 1985). The protein binding affinity, although being a purely drug-specific property, has been reported only in a limited number of cases. It would, however, be of value to determine this parameter more frequently, since the percentage protein binding can be predicted with changing plasma protein concentrations on basis of protein binding affinity. In addition, for highly bound drugs the identification of the protein binding affinity is possibly more accurate than the percentage protein bound due to practical issues (e.g. LOQ). The solubility of newly developed drugs might be an issue in such experiments because high concentrations are needed to saturate albumin binding. This can, however, be solved by adjusting the protein concentration in the solution.

In drug discovery, drug-target binding is defined as affinity- and efficacy-dependent only. In the case of PPB and its influence on PD, capacity, however, is also a major determinant of drug effect. More specifically, the difference in affinity and capacity between target and protein binding determines the influence of PPB on receptor occupancy of a compound under steady-state conditions. In conclusion, capacity is a factor which should be taken into account when assessing the interaction between drug-receptor and drug-protein binding.

We found that the affinity of a drug for its receptor is likely to be higher than for the plasma proteins on individual basis. The ranges in affinities for plasma protein and receptor binding, however, overlapped. As a consequence, it is possible that the affinity of a drug for the protein approaches the affinity for the receptor. Although some studies indicate both restrictive and non-restrictive properties for the PD, it is generally assumed that only the free concentration in plasma is responsible for the pharmacological effect of drugs *in vivo* ('free drug hypothesis') (Wald *et al*., 1992; Van Der Graaf *et al*., 1997; Gerskowitch *et al*., 2007). The simulations showed that under the assumption of rapid-equilibrium, PPB will indeed be restrictive for the PD of most drugs, except for those drugs that have a very high receptor affinity. The active concentration, which is defined as the concentration responsible for effect, is nearer to the free drug concentration than to the total drug concentration. The active concentration, however, does not automatically equal the free drug concentration. Non-restrictive PPB for drugs is possible because of the saturable nature of the target. Non-restrictive binding, however, requires a large difference in the affinity

for protein and target (>1000-fold). In case of a very large difference in the affinity for protein and target, a change in PPB will not result in a shift in the concentration-effect relationship.
The current focus in drug discovery is the search for compounds which display both a high affinity for and a slow off rate from the receptor. Recently, it has been proposed that one of the most crucial factors that determine sustained duration of action is not the affinity of the drug for its target per se, but the residence time of the drug molecule on its target (Copeland *et al*., 2006). NCE are usually aimed to have a high affinity for the physiological target and as a consequence these drugs are likely to have high PPB because of their lipophilicity. Assessment of the influence on PPB on PD is, therefore, of high importance. Compounds with a slow target dissociation rate are believed to have a prolonged duration of action due to this slow dissociation from the target (Dowling and Charlton, 2006). In the present study, the influence of protein binding on PD was only investigated using the assumption of rapid equilibrium (steady state) for both drug-protein and drug-receptor binding. Future research should, thus, determine the impact of PPB on the PD in relation to the binding kinetics at protein and receptor (Talbert *et al*., 2002).

2.7 Appendix A

Linear binding to proteins

Consider the reversible interaction for one compound with a protein (single binding site)

$$[A] + [P] \rightarrow [PA] \qquad \text{(A.1.1)}$$

Under equilibrium conditions we get:

$$\frac{[PA]}{[P][A]} = \frac{1}{Kdp} \qquad \text{(A.1.2)}$$

and

$$PA = \frac{[P][A]}{Kdp} \qquad \text{(A.1.3)}$$

A useful expression is the ratio between the unbound (free) drug and total drug:

$$\varphi = \frac{[A]}{[A] + [PA]} \qquad \text{(A.1.4)}$$

Using equation (A.1.3) we can derive:

$$[A] + [PA] = \frac{[P][A] + Kdp \cdot [A]}{Kdp} \qquad \text{(A.1.5)}$$

Combination of equation (A.1.4) and (A.1.5) now yields:

$$\varphi = \frac{Kdp}{[P] + Kdp} \qquad \text{(A.1.6)}$$

The total protein concentration can be described using the following equation:

$$[P_{tot}] = [P] + [PA] \qquad \text{(A.1.7)}$$

If we assume [*P*]<<[*A*] then [P_{tot}] ~ [*P*] and combination with (A.1.6) yields

$$\varphi = \frac{1}{\frac{[P_{tot}]}{Kdp} + 1} \tag{A.1.8}$$

Non-linear (saturable) binding to proteins

The free fraction can be described on basis of free drug [A] and total drug [D]:

$$\varphi = \frac{[A]}{[A] + [PA]} = \frac{[A]}{[D]} \tag{A.2.1}$$

Combination of (A.1.5) with (A.1.7) yields:

$$[A] = \frac{Kdp \cdot [PA]}{[P_{tot}] - [PA]} \tag{A.2.2}$$

and

$$[A] = \frac{Kdp \cdot [D] - Kdp \cdot [A]}{[P_{tot}] - [D] + [A]} \tag{A.2.3}$$

Rearrangement yields:

$$[A][P] - [A][D] + [A]^2 - Kdp \cdot [D] + Kdp \cdot [A] = 0 \tag{A.2.4}$$

and

$$[A]^2 + [A]([P_{tot}] - [D] + Kdp) - Kdp \cdot [D] = 0 \tag{A.2.5}$$

Solving for [A] gives:

$$[A] = \frac{([D] - [P_{tot}] - Kdp) + \sqrt{([D] - [P_{tot}] - Kdp)^2 + 4 \cdot Kdp \cdot [D]}}{2} \tag{A.2.6}$$

Accordingly:

$$\varphi = \frac{([D] - [P_{tot}] - Kdp) + \sqrt{([D] - [P_{tot}] - Kdp)^2 + 4 \cdot Kdp \cdot [D]}}{2 \cdot [D]} \tag{A.2.7}$$

Simultaneous binding of drugs to protein (unlimited reservoir) and receptor

Consider the reversible interaction for one compound with receptor and protein

[A] + [R] → [RA] (A.3.1)

[A] + [P] → [PA] (A.3.2)

Under equilibrium conditions we get:

$$\frac{[RA]}{[R][A]} = \frac{1}{Kdr} \tag{A.3.3}$$

$$\frac{[PA]}{[P][A]} = \frac{1}{Kdp} \qquad \text{(A.3.4)}$$

Now let the protein reservoir be of unlimited size:

$$\frac{[PA]}{[A]} = \frac{[P]}{Kdp} \qquad \text{(A.3.5)}$$

$$\frac{[P]}{Kdp} = C = constant \qquad \text{(A.3.6)}$$

Let the amount of receptor be:

$$[R_{tot}] = [RA] + [R] \qquad \text{(A.3.7)}$$

The ratio between unbound (free) drug and total drug is:

$$\varphi = \frac{[A]}{[A] + [RA] + [PA]} = \frac{[A]}{[D]} \qquad \text{(A.3.8)}$$

From equation (A.3.3) and (A.3.7) we know:

$$[A] = \frac{Kdr \cdot [RA]}{[R_{tot}] - [RA]} \qquad \text{(A.3.9)}$$

Using equation (A.3.5) and (A.3.6):

$[RA] = [D] - [A] - [PA]$ and $[PA] = C \cdot [A]$

$$[A] = \frac{Kdr \cdot ([D] - [A] - C \cdot [A])}{[R_{tot}] - [D] + [A] \cdot (1 + C)} \qquad \text{(A.3.10)}$$

Rearrangement yields:

$$[A][R_{tot}] - [A][D] + Kdr \cdot [A] + Kdr \cdot C \cdot [A] + [A]^2 \cdot (1 + C) - Kdr \cdot [D] = 0 \qquad \text{(A.3.11)}$$

and

$$(1 + C) \cdot [A]^2 + ([R_{tot}] - [D] + Kdr + C \cdot Kdr) \cdot [A] - Kdr \cdot [D] = 0 \qquad \text{(A.3.12)}$$

Solving for [A] and dividing by total drug [D] to obtain the free fraction (φ) gives:

$$\varphi = \frac{[D] - [R_{tot}] - Kdr - C \cdot Kdr + \sqrt{([D] - [R_{tot}] - Kdr - C \cdot Kdr)^2 + 4 \cdot Kdr \cdot (1 + C) \cdot [D]}}{2 \cdot (1 + C) \cdot D} \qquad \text{(A.3.13)}$$

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