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Review

Drug-protein binding studies New trends in analytical and experimental methodology

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Abstract

In the last few years, continuous progress in instrumental analytical methodology has been achieved with a substantial increase in the number of new, more specific and more flexible methods for ligand–protein assays. In general, the methods used for drug–protein binding studies can be divided into two main groups: separation methods (enabling the calculation of binding parameters, i.e. the number of binding sites and their respective affinity constants) and non-separation methods (describing predominantly qualitative parameters of the ligand–protein complex). This review will be focussed particularly on recent trends in the development of drug–protein binding methods including stereoselective and non-stereoselective aspects using chromatography, capillary electrophoresis and microdialysis as compared to the "conventional approach" using equilibrium dialysis, ultrafiltration or size exclusion chromatography. The advantages and limitations of various methods will be discussed including a focus on "optimal" experimental strategies taking into account in vitro, ex vivo and/or in vivo studies. Furthermore, the importance of some particular aspects concerning the drug binding to proteins (covalent binding of drugs and their metabolites, stereoselective interactions and evaluation of binding data) will be outlined in more detail.

Keywords: Reviews; Drugs; Proteins

Contents

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1. Introduction

The interactions of proteins with various ligands create a basis of an interlocked set of dynamic processes providing a communication and regulation pathway within and between different structures of a living organism. Drug binding to specific plasma transport proteins [albumin (HSA), α_1-acid glycoprotein (AAG), lipoproteins, etc.], is an integral part of many other types of intermolecular interactions in a cellular or organ environment. Different aspects of drug–protein interactions have been reviewed recently, including their molecular nature, biological functions, pharmacological significance as well as methodological approaches applied and their potential shortcomings [1–6]. The objective of this review was to summarize the recent trends in methodologies employed in order to investigate the various aspects of drug–protein interaction and compare them with the so-called "conventional approach".

According to Klotz [7] the techniques used in vitro (or ex vivo) are usually based on one of the following procedures: 1. separation of free and protein-bound fraction of ligand, i.e. determination of the concentration of free ligand; 2. detection of a change in a physicochemical property of the complexed ligand; 3. detection of a change in a physicochemical behaviour of the binding protein. In contrast to non-separation methods, the separation methods allow the study and description of not only the characteristics of primary high-affinity binding sites, but also the (concomitant) presence of secondary low-affinity binding sites. Determination of drug binding to different biomacromolecules, and particularly to specific plasma and tissue proteins, is mandatory in pharmacological and toxicological studies in order to predict nonlinear pharmacokinetic processes [8], stereoselective pharmacokinetics [9], covalent binding of drug metabolites to different molecular structures [10], drug displacement phenomena [11–13], or inter-individual binding variability due to different physiological or pathological factors (age, disease, genetic aspects, etc.) [14–18]. Although the identification of binding structures and the calculation of binding parameters in vitro can provide a very useful quantitative or qualitative information, for a given drug, only combined in vitro and in vivo data can give a complex picture of the impact of binding on its overall pharmacokinetic profile. Recently, some new experimental methodologies, such as microdialysis, have been introduced in the rapidly growing field of ligand–protein binding assays as a promising alternative for measuring of the "true unbound" concentration in vivo in different tissues and body compartments [19,20].

2. Protein–ligand interactions: general considerations

2.1. Reversible binding of drugs and factors influencing drug–protein interactions

Reversible interactions between a protein (P) and ligand (L) can be described according to the law of mass by the following thermodynamic equilibrium:

$$[L] + [P] \overset{k_{1,2}}{\leftrightarrow} [LP] \quad (1)$$

where [LP] is the molar concentration of bound drug, [L] is the molar concentration of ligand, [P] is the molar concentration of protein and k_1 and k_2 ($1/k_1$)

are the rate constants of this interaction. The ratio of the rate constants k_1 and k_2 gives the apparent association constant K_a (l/mol), expressing the affinity of the protein for a particular ligand. By transforming Eq. 1 it is possible to obtain the following equation:

$$B_i = \frac{K_a[L][P]}{1 + K_a[L]} \quad (2)$$

or the commonly used form:

$$B = \sum_{i=1}^{z} \frac{n_i K_{a_i} F}{1 + K_{a_i} F} \quad (3)$$

where B is the concentration of drug bound by a mole of the protein, F is the free drug concentration, K_{a_i} is the equilibrium association constant, n_i is the number of binding sites (identical, independent, i.e. non-interacting) on protein molecule (according to [21]) and z is the number of classes of specific binding sites.

Of course, the interaction of enantiomers of a particular chiral drug ($L_{(S)}$ and $L_{(R)}$) with protein molecule may lead to formation of significantly different complexes ($LP_{(S)}$ and $LP_{(R)}$) (see also Section 2.4) and thus, optical isomers should be seen as essentially different interacting species. Since various stereoselective aspects will be described and discussed in practically all sections of this review, we decided to use symbols (*R*)- and (*S*)- in cases where the absolute configuration of drug enantiomers is known. In some other cases the signs (+)-, resp (−)– were applied. Otherwise, i.e. when the optical activity of chiral drugs is not explicitly indicated by symbols mentioned above, the studied substances were racemates.

This review will be focused particularly on examples concerning the two most important drug transport proteins in plasma, i.e. albumin and α_1-acid glycoprotein. However, the different aspects discussed have much broader plausibility for many other binding proteins (receptors, enzymes, antibodies, ion channel proteins, etc.).

For some drugs, therapeutically achievable concentrations in vivo may saturate the high-affinity binding sites on relevant transport proteins, resulting in a free fraction of drug larger than that observed at lower drug concentrations: the concentration-dependent (nonlinear) binding to plasma proteins has been described for instance for valproic acid [22,23] disopyramide [24,25], cefonicid [26], probenecid [27] or cefixime [28].

Besides changes in drug binding connected with structural alteration of a protein molecule (see Section 2.2 and Section 2.3, the most important changes in the free fraction of the drug are related particularly to disease-induced variations in plasma protein levels: significant, clinically important changes in drug binding have been demonstrated for drugs with hepatic flow dependent extraction [15,16]. Although it seems likely that aging does not have a clinically remarkable impact on protein binding of drugs [14], the genetically determined modifications of proteins exhibit dramatically altered binding behaviour. This was observed in vitro for genetic variants of AAG interacting with psychotropic drugs [18,29,30] or warfarin [18]; the in vivo incidence and significance of these changes is not clear yet. Similarly, a large number of alloalbumins has been discovered [31–33] exhibiting either no change in binding properties [34], or reduced binding affinity due to slight variations in protein conformation [35]. Diurnal variation in concentration of transport proteins (mainly AAG [36]) may also contribute to inter- and intra-individual variability in binding characteristics and should be considered for their accurate interpretation.

Finally, the presence of any "exogenous contaminants" may interfere with drug-protein interactions: the plasticizers tris-(2-butoxyethyl)-phosphate (TBEP) and di-(2-ethylhexyl)-phtalate (DEHP) originating from parenteral therapy devices (e.g. blood transfusion bags, tubes) have been shown to interact in a reversible manner with specific and nonspecific binding sites on β-adrenergic transport proteins including AAG, inhibiting the drug binding on the corresponding sites [37,38]. As it was recently pointed out, the metabolite of DEHP from cigarette filters can be present noncovalently bound to AAG in smokers [39].

2.2. Covalent versus noncovalent binding of drug metabolites to plasma proteins

Many drugs undergo specific metabolic transformation leading to the generation of highly reactive drug intermediates [10]. These reactive forms could:

(1) form covalent or noncovalent bonds to different plasma and tissue macromolecules and/or (2) displace the parent drug from its binding sites. The formation of covalent drug–protein complexes has received increased recognition in the development of a variety of toxic responses [10,40]. The significant degree of binding of metabolites to serum and/or tissue proteins and its potential to displace the parent drug from plasma or tissue binding sites explains, at least for some drugs, the inability to predict accurately drug disposition in vivo from in vitro binding parameters.

Irreversible binding of drugs in form of their acyl glucuronides has been reported for the following interactions: bilirubin–HSA in vitro [41], flufenamic acid–, indomethacin– and benoxaprofen–BSA in vitro [42], etodolac–HSA in vitro [43], zomepirac–HSA in vitro [43], fenoprofen–HSA in vitro and fenoprofen–plasma proteins in vivo [44], tolmetin–plasma proteins in vivo [45] diflunisal–plasma proteins in vivo and in vitro [46] and clofibric acid–BSA in vitro [41] as well as in vivo [47]. Some of these drugs have been withdrawn from the market because of toxic reactions or immunogenic properties.

The mechanisms involved in the binding of drug glucuronides to proteins are multifactorial, particularly in vivo, depending on plasma concentration of the glucuronide and its degradation rate. Thus, a complete knowledge of their nature and consequences connot be considered yet. For example, the covalent attachment of oxaprozine glucuronide [48] or clofibric acid glucuronide [47] to albumin proceeds through the initial formation of a reversible complex most probably at the benzodiazepine binding site. Covalent adducts of clofibric acid have been described in vivo even at concentrations two orders of magnitude less than circulating parent drug concentrations [47]. Although the nature of valproic acid glucuronide binding to serum proteins is not known yet, the discrepancy detected between in vitro binding parameters of valproic acid (two independent classes of binding sites) versus ex vivo binding parameters (one class of binding sites) in rat has led to the assumption that valproic acid glucuronide displaces the parent compound from the high-affinity, low capacity binding site in vivo [49]. A similar mechanism, i.e. increase of the unbound fraction of parent drug due to competitive displacement by metabolite, has been reported for disopyramide [50].

Moreover, metabolic glucuronidation of chiral drugs may be stereoselective [51–53]. As a consequence, formation of diastereomeric glucuronides exhibiting often different protein binding profile could be detected. Since acyl glucuronides are polar and their distribution is restricted to the vascular and interstitial compartments with high concentrations of albumin, the disposition of both glucuronide and pharmacologically active aglycone could be of pharmacological significance. This was demonstrated for instance by Hayball et al. [51] for ketoprofen glucuronides in subjects with impaired renal function. For (*R*)- and (*S*)-oxazepam, stereoselective reversible binding of its diastereomeric glucuronides to HSA has been reported, with no displacing phenomena between parent drug and metabolites (due to ten-fold lower binding affinity of glucuronides) [53]. In contrast to fenoprofen glucuronides exhibiting stereoselective irreversible protein binding both in vitro and in vivo [44], the glucuronides of carprofen [52] or ketoprofen [51] were bound in a reversible stereoselective manner to albumin with the location of binding sites different as compared to the unmetabolized enantiomers. The methodological difficulties for determining the exact protein binding profile of glucuronides are related mainly to their relative instability as well as to the interfering hydrolytic activity of albumin in plasma. Therefore, very rapid separation of bound and unbound fraction should be achieved; details are given in Section 3.

2.3. Binding of ligands to structurally modified proteins

In addition to genetically determined modifications, proteins are commonly a subject of diverse structural changes resulting from the presence of endogenous products of pathologically altered metabolic processes. The changes of proteins induced by different metabolic processes may implicate not only discrepancies in their binding affinity and conformational status, but also an altered uptake mechanism of the protein molecule in target organs or tissues [54,55].

By chronic hyperglycaemia (as observed in poorly controlled diabetes mellitus), the glycosylation of

different plasma proteins has been observed and although hemoglobin has been the most frequently studied marker, examples of proteins undergoing post-translational modification include also HSA or low-density lipoproteins. It has been shown that nonenzymatic glycosylation of HSA in vitro as well as in vivo induces a conformational change in the albumin molecule, and as a consequence, the affinity of various ligands to glycosylated albumin dramatically changes [55]: the affinity of bilirubin reached only about 50% of its value for the nonglycosylated form and the affinity for fatty acids was reduced twenty-fold relative to nonglycosylated albumin. Some studies have suggested that not only the extent of glycosylation, but also some additional factors (e.g. the amount of fatty acids bound per mole of albumin) may affect the changes in drug binding to proteins in diabetic patients [56]. This hypothesis has been supported by clinical experiments which failed to reveal a clear correlation between concentration of glycated albumin and reduced protein binding of phenytoin (75 vs. 81%) or valproate (68 vs. 75%), concluding that the resulting differences in protein binding are due to glucose-independent modification of albumin [57] and likely result from diabetic hyperlipidemia [58].

In uraemia, both structural alterations of the albumin molecule (carbamylation) and accumulation of endogenous compounds seems to be responsible for the observed changes in ligand binding [59]; hippurate, indoxyl sulfate [60] and 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (5-propyl-FPA) [61] have been identified as drug binding inhibitors in human uraemic plasma.

An altered form of albumin with an apparently increased affinity for neurotoxic aluminum has been detected also in Alzheimer's disease [62]: this form of albumin preferentially binds to brain neurons, both facilitating aluminum uptake and impeding magnesium uptake, contributing in this way to the progression of this disease.

2.4. Stereoselective aspects of drug–protein interactions

The plasma binding of racemic drugs is potentially stereoselective, as a consequence of chiral discriminative properties of the binding sites of two important plasma transport proteins, HSA and AAG [63]. Although protein-binding differences (usually up to the factor of 1.5) are not as great as the differences observed between enantiomers in their affinities for receptor structures, they could have important pharmacodynamic and/or pharmacokinetic relevance [64,65].

Because of the vast amount of data published on stereoselective plasma protein binding, it is not possible to discuss all the various drugs. Therefore, only some general comments will be made by presenting the data published on stereoselective plasma protein binding of enantiomers of two representative basic drugs, propranolol and verapamil (Table 1 and Table 2) and binding parameters obtained for binding of two chiral acidic NSAIDs ((*RS*)-, (*R*)-, (*S*)-carprofen and (+)-, (−)–pirprofen) to human serum albumin (Table 3). It is quite clear that many factors (physiological factors including interspecies binding differences, methodological approach used, enantiomer–enantiomer interactions at binding sites, etc.) could significantly influence the interpretation of binding results.

The recognition of eventual stereoselective plasma binding differences between humans and other species commonly used in pharmacokinetic studies (especially rat and dog) is of utmost importance, since there is always a need to extrapolate the experimental data in order to predict the situation in man. Besides the enantiomers of propranolol [66–68] and verapamil [69,70], the stereoselective differences in protein binding between different species have been described also for the optical antipodes of warfarin [71], ofloxacin [72], leukotriene D_4-antagonist, MK-571 [73], carbonic anhydrase inhibitor, MK-927 [74] and disopyramide [75].

The interspecies binding differences of (*R*)- and (*S*)-propranolol are related mainly to opposite stereoselectivity found between rat and human (or dog) (Table 1). On the other hand, the oral kinetics of propranolol enantiomers has been found to be opposite between dog and man [76]. Takahashi et al. [68] considering a competitive binding of individual propranolol enantiomers in rat have pointed out, that particularly the differences in the affinity (K_a) of the specific binding sites in rat plasma seem to be responsible for the higher percentage of bound (*R*)-propranolol as compared with (*S*)-propranolol. Simi-

Table 1
Stereoselective protein binding of propranolol enantiomers in rat, dog and man

Species	Protein fraction	Dose or drug concentration added	f_u[a]		Experimental conditions	Reference
			(*R*)-Propranolol	(*S*)-Propranolol		
Rat	Plasma ex vivo	10 mg/kg[c] p.o.	0.038±0.003	0.213±0.018	ED[b], 4 h, 37°C	[89]
	Serum in vitro	50 ng/ml[c]	0.068±0.014	0.261±0.010	ED, 4 h, 37°C	[78]
	Plasma in vitro	500 ng/ml[d]	0.046	0.101	Ultrafiltration 37°C	[68]
Dog	Plasma in vitro	150 ng/ml[d]	0.167±0.020	0.130±0.020	ED, 18 h, 37°C	[67]
Human	AAG (0.66 g/l) in vitro		0.162±0.017	0.127±0.013		
	HSA (40 g/l) in vitro	125 ng/ml[d]	0.607±0.013	0.649±0.011	ED, 10 h, 37°C	[233]
	Plasma in vitro		0.253±0.019	0.220±0.020		
	AAG (1.10 g/l) in vitro		0.302±0.9	0.230±0.030		
	HSA (40 g/l) in vitro	100–120 ng/ml[d]	0.482±0.016	0.510±0.090	ED, 1.5 h, 37°C	[66]
	Plasma in vitro		0.122±0.190	0.109 ± 0.020		

AAG, human α_1-acid glycoprotein; HSA, human serum albumin.
[a] f_u, unbound fraction.
[b] ED, equilibrium dialysis.
[c] Administration or addition of drug in form of racemate.
[d] Administration or addition of drug in form of individual enantiomers.

larly, for binding of (*R*)- and (*S*)-disopyramide in plasma, it has been shown that the interspecies binding variability is related particularly to differences in K_a-values of individual enantiomers [75,77].

For (*RS*)-verapamil, the finding about the preferential binding of (*R*)-enantiomer in dog [70] as opposed to rat [78] mimics also the binding profile observed in man [69]. Nevertheless, although the apparent volume of distribution of (*S*)-verapamil has been found to be significantly greater than that of (*R*)-verapamil in both dog and man, the reason appears to be different: in man, it is probably related to a lower plasma binding of (*S*)-verapamil, while in dog, the mechanism appears to be related to a higher tissue binding of (*S*)-verapamil [70,79].

Protein (and particularly albumin) binding charac-

Table 2
Stereoselective protein binding of verapamil enantiomers in rat, dog and man

Species	Protein fraction	Dose or drug concentration added	f_u[a]		Experimental conditions	Reference
			(*R*)-verapamil	(*S*)-verapamil		
Rat	Serum in vitro	50 ng/ml[c]	0.169±0.011	0.075±0.005	ED[b], 4 h, 37°C	[78]
Dog	Plasma ex vivo	500 ng/ml[c]	0.28±0.05	0.32±0.02	ED, 18 h, 37°C	[70]
Human	AAG (0.55 g/l) in vitro		0.079±0.016	0.142±0.020		
	HSA (40 g/l) in vitro	0.055–22 μg/ml[d]	0.400±0.030	0.572±0.029	ED, 16 h, 37°C	[69]
	Serum in vitro		0.096±0.009	0.136±0.006		
	Serum ex vivo	80 mg p.o.[d]	0.13±0.02	0.23±0.03	ED, 16 h, 37°C	[69]
	Serum ex vivo	5 mg i.v.[d]	0.06±0.01	0.12±0.02		
	Plasma in vitro	10–500 ng/ml[d]	0.063±0.022	0.115±0.016	ED, 4 h, 37°C	[79]
	Plasma in vitro	[d](*R*)-:100 ng/ml [d](*S*)-:1 μg/ml	0.043	0.108	ED, n.a.	[87]

AAG, human α_1-acid glycoprotein; HSA, human serum albumin; n.a., not available.
[a] f_u, unbound fraction.
[b] ED, equilibrium dialysis.
[c] Administration or addition of drug in form of racemate.
[d] Administration or addition of drug in form of individual enantiomers.

Table 3
Binding parameters of chiral NSAIDs bound to HSA as determined by different experimental techniques in vitro

Drug	n_1	K_{a1} ($\times 10^6$ l/mol)	n_2	K_{a2} ($\times 10^5$ l/mol)	Albumin concentration (μmol/l)	Method/temperature	Reference
(*RS*)-Carprofen	0.9±0.1	5.1±0.2	7.3±0.3	3.7±0.3			
(*S*)-Carprofen	1.1±0.1	5.3±0.2	8.7±0.3	1.7±0.1	20 or 2	Fluorescence,	[184]
(*R*)-Carprofen	0.8±0.1	4.7±0.2	7.9±0.4	4.6±0.3		25°C	
(*RS*)-Carprofen	1.2±0.2	3.7±0.3	4.0±0.3	1.3±0.1			
(*S*)-Carprofen	1.3±0.1	4.7±0.2	4.5±0.3	1.4±0.1	50	ED[b]	[98]
(*R*)-Carprofen	1.1±0.1	3.5±0.2	4.8±0.4	1.4±0.1		25°C	
(*S*)-Carprofen	2.12±0.07	1.08±0.07	0.06±0.01[a]			Ultrafiltration,	[52]
(*R*)-Carprofen	1.80±0.06	0.73±0.04	0.07±0.00[a]		30	ambient	
(±)-Pirprofen	0.90±0.30	0.39±0.1	2.90±0.90	0.08±0.04			
(+)-Pirprofen	0.90±0.20	0.42±0.2	3.40±0.20	0.10±0.03	50	ED	[206]
(−)-Pirprofen	0.90±0.20	0.41±0.1	3.50±0.10	0.11±0.02		n.a.[c]	
(+)-Pirprofen	1.91±0.13	0.41±0.06	0.15±0.02[a]			HPLC	
(−)-Pirprofen	2.07±0.13	0.66±0.14	0.09±0.01[a]		15	37°C	[230]

[a] Non-specific binding, i.e. the product of $n \times K_a$ ($\times 10^5$ l/mol).
[b] ED, equilibrium dialysis.
[c] n.a., data not available.

teristics are primary determinants of the pharmacokinetics of 2-arylpropionic acid derivatives, profens. Their primary high-affinity binding site on albumin, i.e. site II (benzodiazepine site) manifests more impressive stereoselective behaviour in comparison to site I (warfarin site). Some of them are bound stereoselectively (enantiomers of ketoprofen [80], ibuprofen [81,82], flurbiprofen [83]) and others are not ((*R*)- and (*S*)-fenoprofen [84]). Table 3 illustrates the binding differences observed in vitro for interaction of carprofen and pirprofen enantiomers with HSA as measured by different experimental methods. The remarkable differences described for binding parameters of individual enantiomers by different authors could be explained not only by different albumin concentrations used, but also by different experimental conditions applied (the influence of temperature is in this particular case not significant). This important source of confusion in literature concerning the stereoselective binding studies is further discussed in Section 3.1, Section 3.2, Section 3.3 and Section 3.4; the significance of knowledge of diastereomeric glucuronides binding characteristics is mentioned in Section 2.2 of this review.

Considering some other factors, the stereoselective protein binding does not appear to be influenced by age. This has been supported by findings observed in man for enantiomers of propranolol [85,86], verapamil [87], disopyramide [88] and is consistent also with the reports about stereoselective propranolol binding in rat [89]. On the other hand, as it has been demonstrated by Eap et al. [17] for (+)- and (−)-methadone, genetic variants of some human proteins (AAG) may interact in a different way with individual drug enantiomers. Consistently, Herve et al. [18] have supposed, based on their rather surprising experimental results with separate gene variants of AAG ($n = 0.6$), a chiral recognition of the two genetic forms of AAG (i.e. F1 and S) for (*RS*)-warfarin. However, at present little is known about possible phenotype-dependent stereoselective protein binding.

A very important point from the generation and interpretation of binding results originating especially from nonstereospecific assays is represented by potential enantiomer–enantiomer interaction at the relevant plasma protein binding sites [90].

First, a competitive inhibition mechanism may be considered, as it was the case of (*R*)- and (*S*)-disopyramide interaction with human AAG binding site(s) both in vitro [16] and in vivo [91] as well as for disopyramide enantiomer interaction with mono-N-dealkyldisopyramide [92]. Similarly, the enantiomers of flurbiprofen exhibited a mutual competition by saturating the available plasma binding sites in rat [93]. As pointed out by Honore [94], competitive binding of ligands to albumin may also involve very

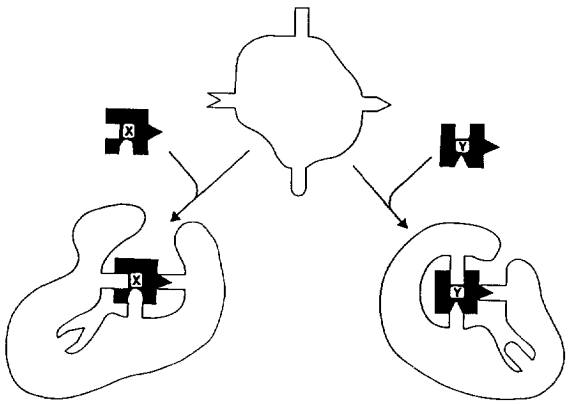

Fig. 1. Competitive binding of two ligands X and Y to albumin considering the "induced fit" model: the ligands are bound in a competitive manner although they do not interact with the same site. Slightly modified representation according to Honore [94].

different parts of its primary structure, i.e. two ligands could bind competitively, although in molecular terms they do not bind to the same site (Fig. 1). In this "induced fit" model each of the two binding sites is represented not by a preformed rigid structure, but by conformational changes of the protein molecule induced by binding of each of the two ligands. The plausibility of such a model for some of the stereoselective binding interactions remains to be established.

Second, allosteric displacement has been reported to be responsible for stereoselective displacement of benzodiazepines by (*S*)-warfarin [95] or (*S*)-phenprocoumon [96]. Noctor et al. [97] have suggested that the actual mechanism involved may depend on the concentration of the displacer: binding of a model displacer (octanoic acid) to its primary binding site I on albumin induced a conformational change in the micro-environment of drug binding site II, allosterically displacing the drugs bound there (e.g. (±)-suprofen, (*RS*)-ketoprofen). On the contrary, at higher concentrations of displacer, a competitive displacement at site I has been observed.

Most recently, the so-called stereoselective site-to-site displacement of protein-bound drug has been described by Rahman et al. [98]. Based on the association constant values, the binding without competitor was of the following order: (*S*)-(+)- > *RS* (±) > (*R*)-(−)- and (*R*)-(−)- > *RS* (±) > (*S*)-(+)- for carprofen and ibuprofen, respectively. In the presence of (*R*)- and (*S*)-ibuprofen, carprofen

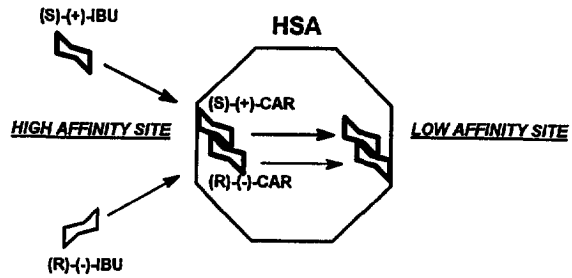

Fig. 2. Stereospecific site-to-site displacement of (*R*)- and (*S*)-carprofen bound to HSA by the enantiomers of ibuprofen. Slightly modified representation according to Rahman et al. [98]).

enantiomers were displaced from their high-affinity binding site (site II) to their low affinity binding site (site I) (Fig. 2). When this site was blocked by a sufficient amount of either (*RS*)-warfarin or phenylbutazone, a complete displacement of caprofen enantiomers was achieved resulting in almost four-fold increase of their free fraction. In addition, although on the basis of K_a-values the interaction between (*R*)-carprofen (3.5×10^6 l/mol) and (*R*)-ibuprofen (3.0×10^6 l/mol) had to be the strongest, it was not the case. The facts about the different association constants and number of binding sites observed for (*R*)- and (*S*)-ibuprofen in the most recent study of Hage et al. [99] have further confirmed the importance of the study of individual enantiomeric differences in the development of an objective picture of drug–protein interaction.

3. Methodology of in vitro and ex vivo binding experiments

3.1. Conventional methods

From this group of methods, equilibrium dialysis and ultrafiltration are undoubtedly the most widely used because of their simplicity and general applicability to many different systems in vitro and ex vivo. Equilibrium dialysis is based on establishment of an equilibrium state between a protein compartment and buffer compartment which are separated by a membrane which is permeable only for a low-molecular-weight ligand. On the other hand, ultrafiltration with semipermeable membranes produces a separation of the free drug from macromolecules by employing a pressure gradient which forces the small

molecules through the membrane. The possible interfering factors and shortcomings of equilibrium dialysis and ultrafiltration as a membrane separation method, as compared to ultracentrifugation, another conventional separation method, are summarized in Table 4.

Although there is no "standard method" for binding measurements, equilibrium dialysis is often regarded as the "reference method" for the determination of drug–protein binding profile. However, no available experimental data support this supposed superiority, particularly since this method has many problems, including the time needed to reach equilibrium [100,101], volume shifts [102], Donnan effects, hindering of the passage of free ligand [103], nonspecific adsorption to dialysis apparatus [104] and difficulty in the control of some experimental variables (pH of dialysate, [105–107]). The adsorption of ligands to the surface of the dialysis device and dialysis membrane is a serious problem particularly for highly lipophilic drugs: for example, for cyclosporin the use of steel chambers has been reported, instead of Teflon or Perspex cells which exhibit extensive adsorption (98%) of this compound [108]. Furthermore, when a radiolabeled drug is used for binding measurements the observed free fraction may be overestimated as a result of presence of radiochemical impurities and/or nonstability of the labeled ligand [109,110]. Since the radiochemical impurity associated with the ligand could act also as an effective inhibitor of binding [111] and the presence of radiolabel impurities is relatively common in ^{3}H-labeled compounds, it is likely that many published binding data neglecting the above mentioned fact may become obscured. Therefore, it is suggested that the radiolabel should be purified prior to dialysis and also that its postdialysis stability should be checked. Additionally, significant overestimation of the free fraction can result from even slight leakage of protein (or fragments of it) into the dialysate [101] and thus, the postdialysis stability of protein and its absence from the dialysate should be confirmed by protein assay in a validation study.

Ultrafiltration has been introduced widely for routine free drug monitoring in clinical laboratories, since it offers significant advantages represented by short analysis time, simple, commercially available kits (Centrifree Micropartition System from Amicon or Molcut II from Millipore) and lack of dilution effects and volume shifts. The major controversy involves the stability of the binding equilibrium during the separation process. It is therefore advisable to validate, especially in case of low-affinity interactions, the basic assumption that the binding ratio of protein-bound drug to free drug remains constant. Rather exceptionally, the loss of drug [112] or protein during separation process has been reported.

Ultracentrifugation is an alternative to both equilibrium dialysis and ultrafiltration since it eliminates the problems associated with membrane effects and enables the separation of the free and protein-bound fraction in a "natural environment", i.e. without addition of buffer systems and therefore, dilution problems. However, several comparative binding studies with different types of drugs have revealed that there are quantitative discrepancies between results obtained by equilibrium dialysis (or ultrafiltration) as compared to ultracentrifugation [100,113–115]. As it was pointed out, the error in the estimation of the free drug concentration can be influenced by physical phenomena such as sedimentation, back diffusion, viscosity and binding to plasma lipoproteins in the supernatant fluid. The possible floating of lipoprotein fractions interfered mainly with the determination of binding characteristics of basic drugs [115].

Recently, some systems based on equilibrium dialysis have been introduced for tissue binding studies (distribution dialysis procedure, [116,117]) or ASTED (automated sequential trace enrichment of dialysate) for routine monitoring of free fractions of drugs in biological fluids, combining on-line equilibrium dialysis, trace enrichment and HPLC in a column-switching system [118]. Although these methods exhibited some improvements (increased dialysis rate, i.e. shorter analysis time in system ASTED), the influence of membrane effects is still a limiting factor.

3.2. Chromatographic methods

Despite the fact that chromatographic methods have been used for a long time for the determination of drug–protein binding parameters, they have earned only limited attention. Conventional size-exclusion columns exhibited low efficiencies, poor

Table 4
Comparison of conventional separation methods

Interfering factors	Equilibrium dialysis	Ultrafiltration	Ultracentrifugation
Equilibrium time	Can be long (up to 20 h)	Short (10–15 min)	Usually long (12–15 h)
Amount of sample	Usually 500–1000 μl	Small (<1 ml), depending on K_a of the interaction	Usually >1 ml
Temperature control	Available	Usually not available	Usually not available
pH control	Necessary	Not necessary	Necessary
Donnan effects	Yes	Yes	No
Adsorption to membranes and apparatus	Yes	Yes	No
Dilution of the sample: volume shifts	Yes	No	No
Membrane permselectivity	Presence of "sieve" effects	Usually satisfying	–
Other shortcomings of the method	Not suitable for drugs undergoing hydrolysis in serum	Stability of the free fraction (i.e. the binding ratio of protein-bound drug to unbound protein) may exhibit changes during the separation process	Binding equilibrium may be altered during separation process (sedimentation, back diffusion)
	Changes in stability of ligand and protein may occur	Leakage of drug or protein	Expensive equipment

protein recoveries and short column life-times and therefore, the methods were experimentally unconvenient and time consuming. The progress in chromatographic technology has led to the development of highly automated systems yielding high resolution on small columns, allowing shorter analysis times, consuming less chemicals and avoiding the use of radiolabeled ligands. In general, binding data obtained by using chromatographic methods offer much higher precision and reproducibility than those measured by conventional techniques, and also provide the possibility to detect very small differences in the binding affinity of ligands (e.g. structural derivatives or enantiomers).

3.2.1. Affinity chromatography: use of protein stationary phases (PSPs)

Although affinity chromatography has been introduced mainly for the isolation and purification of biologically active compounds, the introduced HPLC stationary phase materials with immobilized biopolymers (enzymes, receptors, ion channels, or antibodies) provided, in addition, a powerful tool for studying the interactions between small ligands and biomacromolecules [97,119,120].

The advantages of this approach are represented particularly by the stability and constant binding behaviour of immobilized biopolymers, the precision and reproducibility of the chromatographic system (enabling large sets of comparative binding studies), effectiveness by using small amounts of ligands (enantiomers) and the ability to perform enantioselective studies even if the enantiomers racemize in aqueous media [119]. Since non-physiological experimental conditions (pH adjustment, presence of organic modifiers) may alter the conformation and the natural binding behaviour of the protein, it is necessary to take these factors into account during the interpretation of results. Assuming that the immobilization procedure does not influence the binding properties of protein, the studies based on the use of PSPs may provide information on extent (i.e. relative affinity) of ligand binding and on the area(s) where the interaction takes place. Moreover, this methodology allows the study of the enantioselective protein-binding phenomena as well as drug–drug or enantiomer–enantiomer interactions which are difficult to evaluate by conventional methods since they require the determination of the isomeric composition of the equilibrium mixture (by use of pseudoracemates or enantioselective chromatographic techniques).

Studies with 1,4-benzodiazepine derivatives [121–124], 2-arylpropionic NSAIDs [125], coumarin derivatives [96,126,127], vinca alcaloids [128] triazole derivatives [127], etc. (Table 5) have confirmed that

Table 5
Immobilized proteins (PSPs) used as probes of protein-ligand interactions in affinity HPLC

Protein	Immobilization procedure/column[a]	Ligand	Reference
HSA	(1)	Acenocoumarol, warfarin	[126]
	(1)	3-Acetoxy- and 3-hydroxy-1,4-benzodiazepines, warfarin	[121]
	(1)	Vinca alkaloid analogues	[128]
	Chiral-protein-2 (2)	1,4-benzodiazepine derivatives	[123,124]
	Chiral-protein-2 (3)	Oxazepam	[234]
	(1)	[^{125}I]Thyroxine	[235]
	(4)	Warfarin, benzodiazepines	[122]
	(5)	Warfarin enantiomers	[236]
	(4)	2-Arylpropionic acid NSAIDs	[125]
HSA(recombinant)	(1)	1,4-Benzodiazepine derivatives; coumarine derivatives	[237]
BSA	Crosslinking of BSA to 100A Kromasil 7μ	α-Aryl- and α-aryloxy alkanoic acids (profens); α-N-(N′-carbazolcarbonyl)-amino acids	[130]
AGP	EnantioPAC (LKB)	(*S*)-Propranolol; other model drugs	[132]

[a](1) HSA immobilized on CNBr activated Sepharose; (2) commercially available HSA-CSP (Shandon Scientific:PLC); (3) commercially available HSA-CSP (Societe Francaise Chromato Colonne); (4) HSA immobilized on 1,1-carbonyl diimidazole activated Diol-HPLC column, (5) HSA immobilized onto the diol-bonded Nucleosil using the Schiff base method.

HSA–PSP accurately reflects the binding behaviour of non-immobilized (free) HSA including its "native enantioselectivity". As a consequence, HSA–PSP has been successfully used as a quantitative probe of drug binding to albumin (when the binding is ≥60%) as well as a qualitative probe for drug–drug interactions (non-cooperative, cooperative interactions and independent binding). Previously reported differences [129] in the binding behaviour of HSA and BSA cannot be confirmed further [125,130], with the exception of the reversed elution order of the enantiomers of warfarin on HSA–PSP ((*R*)- before (*S*)-warfarin) as compared to BSA–PSP.

Contrary to the behaviour of albumin, the immobilized α_1-acid glycoprotein is not suitable as a screening tool for AAG binding affinity. Although the system has been widely used to separate enantiomers of different compounds (usually with addition of organic modifiers and pH adjustment [131]), there was a lack of strong correlation between the retention of compounds known to bind to AAG and their potential to displace (*S*)-propranolol as a high-affinity binding marker [132]. The possible explanation outlined by Schill [133] takes into account the fact that a number of the carboxylic groups of sialic acid are tied up in the ionic bonding with the silica support and are therefore not available for interaction with positively charged drugs. Although the removal of sialic acid residues is not accompanied by a conformational alteration of native AAG [134], asialoAAG exhibited significantly lower affinity for cationic ligands [135–137]. As a consequence, the immobilized protein retains in part its binding properties for cationic compounds, however, the affinity of the relevant binding site(s) differs from those of the native protein. Haginaka et al. [138] have recently proposed that the chiral recognition ability of ovomucoid originates particularly from the fraction termed ovoglycoprotein (with reduced sialic acid content) present in the commercial and isolated ovomucoid preparations as an impurity.

Most recently, Aubry et al. [139] have introduced a chiral stationary phase based on a mixture of immobilized HSA and AAG. Interestingly, a "cooperative binding effect" of these two proteins has been described for interaction with (*RS*)-lidocaine [140] or (*RS*)-propranolol [141]. The use of mixed PSPs for evaluation of more complex binding interactions is questionable.

3.2.2. High-performance size-exclusion chromatography

Several variants of high performance size-exclusion chromatographic techniques for binding interactions have been described: frontal analysis (HPFA) [142], vacancy peak method [143,144], retention analysis [145–147] and the Hummel–Dreyer method [142,144,148].

Frontal analysis allows direct sample injection and is, together with the Hummel–Dreyer method, considered as an equilibrium technique enabling the quantitative description of binding parameters of various types of ligands. Recently, both methods were further optimized by Pinkerton by the introduction of the internal surface reversed-phase (ISRP) type "restricted access" columns [149,150], specifically designed to facilitate the HPLC analysis of drugs in plasma or serum by direct injection. The ISRP combines the principles of size exclusion and bonded phase partitioning separations having hydrophilic diol-glycine groups on the external surface which eliminating protein adsorption, and a tripeptide partitioning phase (Gly-Phe-Phe) on the internal surface of pores. Drugs of low-molecular weight are able to enter the pores of the ISRP column and partition with the tripeptide phase unlike large protein molecules, eluting in the column interstitial void volume.

In the method of Hummel and Dreyer ([151], schematically presented in Fig. 3a) the column is equilibrated with a mobile phase containing a given concentration of drug in phosphate buffer. When the drug–protein complex is injected, equilibrium between ligand and protein is rapidly reestablished in accordance with the free ligand concentration in the mobile phase: the protein and drug–protein complex elute in the column interstitial void volume and the ligand migrates according to its pore volume penetration and distribution in the stationary phase. For the quantification of the amount of drug bound, internal or external calibration procedures have been adopted (Fig. 3b; [142,152]). If a column meets the requirements of nonadsorptive, size-exclusion of proteins with concurrent chromatographic partitioning of the ligand (e.g. diol- or ISRP columns), the bound concentration can be measured directly, while the free ligand concentration is controlled in the mobile phase as the true independent variable [153]. As pointed out by Pinkerton and Koeplinger [153], this

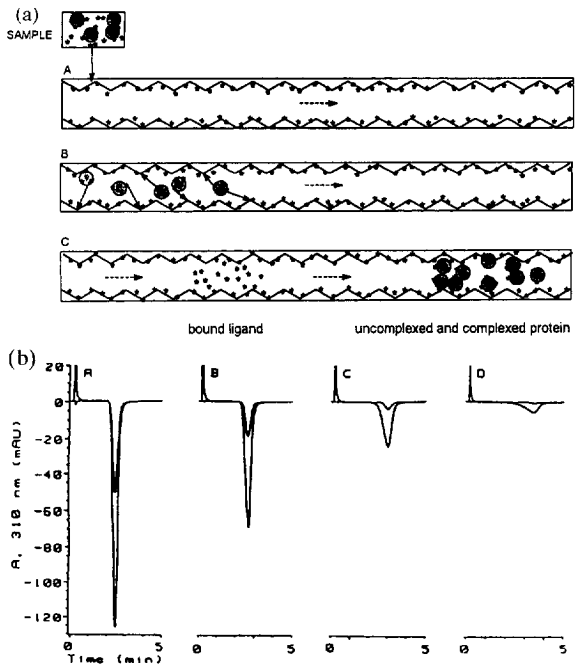

Fig. 3. (a) Schematic presentation of the Hummel–Dreyer method in HPLC conditions by injecting excess of drug in the sample as compared to drug concentration in the mobile phase. (A) establishing of the equilibrium at the beginning of the sample injection; (B) second equilibrium after the sample injection; (C) separation of drug–protein complex and "bound" drug. (b) Representative Hummel–Dreyer elution profiles for (*RS*)-warfarin–HSA binding. Reprinted, with permission, from Pinkerton and Koeplinger [153]). HPLC conditions: ISRP column (5 cm × 4.5 mm I.D.); (*RS*)-warfarin mobile phase concentration in 0.067 mol/l phosphate buffer (pH=7.4): (A) 81.1 μmol/l, (B) 32.5 μmol/l, (C) 8.1 μmol/l, (D) 1.6 μmol/l; flow-rate: 2 ml/min; detection: 310 nm. Top trace is injection of buffer blank and bottom trace is from injection of HSA.

is the unique feature of this method — all other methods require that the total drug concentration is controlled and the free drug concentration measured. In addition, although the protein throughout the sample may differ in concentration, it is permanently equilibrated with free drug in the mobile phase and therefore, the "on-column dilution effect" is of no consequence [153]. Obviously, this method provides an efficient means of determining accurate binding parameters for protein–ligand equilibria occuring at more than one type of binding site (concomitant presence of high- and low-affinity binding sites) [152,154] and it could be adopted also in case of highly protein bound hydrophobic drugs [155,156] or for evaluating of stereocontrolled binding differences of individual drug enantiomers [154,156–158]. Moreover, it is suitable for further automation [153]. The most important disadvantage of this method is related to the restriction that only purified protein or biopolymer samples may be used.

In the frontal analysis method (HPFA, Fig. 4a, Table 6), a large volume of drug–protein mixed solution is applied continuously onto a size-exclusion column to achieve a steady-state concentration. The elution profile obtained consists of α-, β- and γ-plateau zones corresponding to the free protein, mixture of protein-bound ligand and free ligand, respectively (Fig. 4b). The major limitation of this method is that a large volume of sample is needed to observe a clear γ-plateau which represents the free drug concentration. In HPFA with μBondagel column (30 cm × 3.9 mm I.D., 5–10 μm), the use of an 18-ml sample of warfarin–HSA has been reported [142]; by using the ISRP silica column (10 cm × 4.6 mm I.D., 5 μm) the injection volume for warfarin–BSA could be reduced to 10 ml [159]. However, when the drug is more hydrophobic (and is retained to the ISRP silica support more strongly), frontal analysis can be performed with a shorter column and consequently, with a smaller sample volume (100 μl–2 ml, depending on the free drug fraction) [160]. This makes the HPFA method advantageous particularly for highly bound hydrophobic drugs with strong adsorption effects that interfere when the conventional separation approach is used. Applying a non-stereoselective assay, a good correlation between binding characteristics by using this method ($n = 1.46$; $K_a = 2.44 \times 10^6$ l/mol) as compared to ultrafiltration–HPLC method ($n = 1.47$; $K_a = 2.34 \times 10^6$ l/mol) has been reported for fenoprofen [161]. Furthermore, the HPFA coupled with a chiral-HPLC system offers the prospect of direct and enantioselective quantification of unbound drug at protein-binding equilibrium, as it has been shown by Shibukawa and coworkers for warfarin [162], ketoprofen [163], fenoprofen [161] and nilvadipine [164].

3.3. Capillary electrophoresis

Because of its speed, efficiency and selectivity, capillary electrophoresis (CE)is currently the most dynamically growing analytical technique in separation methods. CE is a powerful complementary

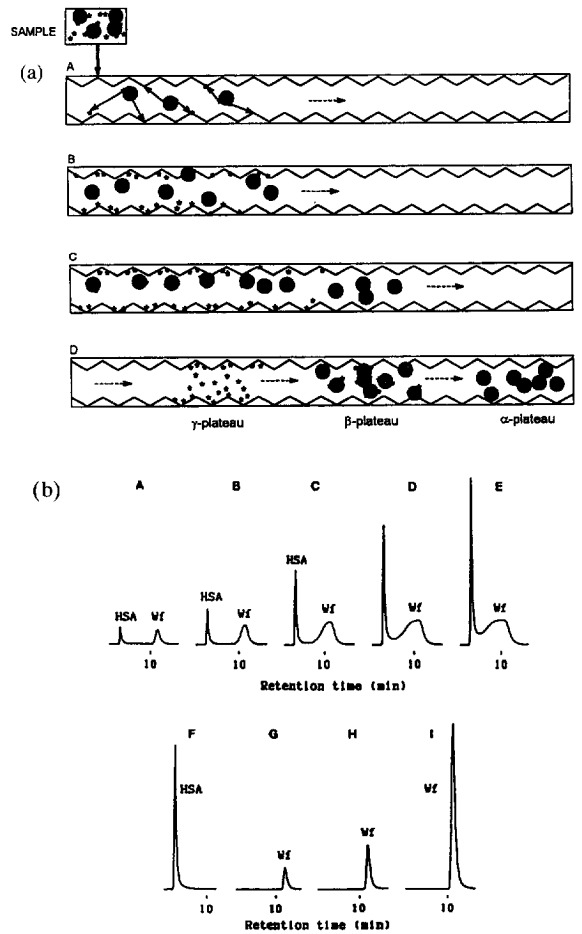

Fig. 4. (a) Schematic diagram of the frontal analysis method in HPLC conditions. Slightly modified representation according to Shibukawa et al. [231]. (A) beginning of the sample injection; (B) establishing of equilibrium in the interstice of the stationary particles; (C) end of sample injection; (D) separation of uncomplexed protein (α-plateau), drug–protein associate (β-plateau) and free drug (γ-plateau). (b) High-performance frontal analysis of (*RS*)-warfarin(Wf)–HSA interaction. Reproduced with permission from [162]. Injected samples: 200 μmol/l Wf–550 μmol/l HSA (A–E), 550 μmol/l HSA (F), and 200 μmol/l Wf (G–I). Injection volume: (A,G) 5 μl; (B,H) 10 μl; (C) 20 μl; (D) 30 μl; (E,F,I) 40 μl. HPLC conditions: ISRP silica column (15 cm × 4.6 mm I.D.) thermostated on 37°C; mobile phase: potassium phophate buffer (pH=7.4), flow-rate 0.5 ml/min; detection: 308 nm). The absorbance units full scale of chromatograms A and B was four times larger than that of chromatograms C–G.

tool to both conventional gel electrophoresis and HPLC in several biomedical applications [165]. Most recently, various forms and modifications of CE, including affinity capillary electrophoresis (ACE) [166–169], capillary affinity gel electrophoresis (CAGE) [170–172] or packed-capillary electrochromatography (CEC) with immobilized protein–stationary phase [173], have been used to characterize the binding of ligands to proteins (representative results are summarized in Table 7).

Similarly, as in chromatography, CE offers several possibilities for studying ligand–protein binding interactions. In principle, if the protein adsorption to the capillary surface is not significant, there are two types of experimental procedures [174,175], both involving the measurement of changes in mobility of the protein as a function of the concentration of a ligand (or ligands) in electrophoresis buffer. First, when a protein forms a complex with a (charged) ligand of relatively small mass, the change in the electrophoretic mobility of the complex is large as compared to the change in its mass. If the protein–ligand complex has a measurable difference in electrophoretic mobility relative to the free protein, Scatchard analysis of the change in the electrophoretic mobility of the protein as a function of the concentration of the ligand allows the direct determination of the binding constant of the interaction, K_a. Secondly, a variant of this method (injection of a mixture of two ligands: one charged and one electrically neutral) enables the measurement of the binding constant of a neutral ligand. Both types of procedures are best carried out by measuring changes in migration time relative to another non-interacting protein having a similar value for migration time. However, if stereoselective aspects are considered, individual enantiomers are necessary to evaluate the eventual binding differences.

Another, complementary type of experiment is represented by using the binding protein as a buffer additive in ACE [166,168,169,176,177]. The advantage of this approach is that the injected ligand may be a racemate, and if stereoselective binding occurs, a chiral separation could be achieved. In this way, Arai and coworkers [176,177] investigated the stereoselective interaction of ofloxacin with different albumins (either chemically modified or from different biological species). Consistent with stereoselective species-dependent protein binding of this drug [72], they were able to describe varying chiral discriminative capacities of isolated animal albumins. The most interesting finding was related to stereoselective ofloxacin–BSA vs. nonstereoselective

Table 6
High-performance frontal analysis method (HPLC-FA) for determination of drug-protein interaction

Protein	Ligand	HPLC column	Results	Reference
HSA	(*RS*)-Warfarin	HPFA: μBondapac column	Binding parameters: n, K_a [a]	[142]
BSA	(*RS*)-Warfarin, indomethacin	HPFA: ISRP silica column	Free drug concentration [b]	[159]
Human plasma	Carbamazepine	HPFA: ISRP silica column	Simultaneous determination of free and total drug concentration [b]	[231]
HSA, BSA	Salicylate, acetazolamide	HPFA: hydrophilic polyvinyl alcohol gel (Asahipak GS-320)	Estimation of salicylate binding parameters (n, K_a) [b]	[238]
HSA	(*RS*)-Warfarin	HPFA (ISRP silica)→Chiral AGP	Free concentrations of (*R*)- and (*S*)-warfarin [b]	[162]
HSA, human plasma	(*RS*)-Ketoprofen	HPFA (Hisep column)→preconcentration column→Sumichiral OA2500S (Pirkle-type) column	Enantioselective determination of unbound drug concentration (detection limit: 1.0 n*M*) [b]	[163]
HSA	(*RS*)-Fenoprofen	HPFA: Pinkerton column→Chiral AGP	n, K_a of (*RS*)-fenoprofen; enantioselective determination of unbound drug [b]	[161]
HSA	(*RS*)-Nilvadipine	HPFA: diol-silica column (Develosil 100 Diol-5)→extraction column→ovomucoid-immobilized HPLC column	Enantioselective determination of unbound drug (det. limit: 0.1 n*M*) [b]	[164]

[a] Compared with the Hummel and Dreyer method.
[b] Results validated by conventional ultrafiltration-HPLC method.

Table 7
Capillary electrophoresis methods for protein-ligand binding studies

Protein	Drug–ligand	Method	Experimental design	Reference
BSA	D- and L-tryptophan	CAGE	Protein is crosslinked with glutaraldehyde	[170]
	(6*R*)- and (6*S*)-leucovorin	ACE	P-BA	[166]
	(6*R*)- and (6*S*)-leucovorin	ACE	Protein is covalently bound in form of a dextran-protein network	[179]
	(*RS*)-Warfarin	HDM, VPM, FA	P-BA (VPM) or the mixture of protein and ligand is injected(FA, HDM)	[180]
BSA Palmitic-, glucosamide-, with respect to acetyl-BSA, albumins from different species	(±)-Ofloxacin, DL-Tryptophan	ACE	P-BA; (evaluation of stereoselective binding aspects); investigation of displacing interactions	[177]
BSA, HSA	(±)-Ofloxacin and its derivative DR-3862, (*RS*)-Warfarin	ACE	P-BA	[176]
HSA, AGP	(*RS*)-Verapamil	FA	Mixture of protein and ligand is injected	[160]
HSA	(*R*)- and (*S*)-verapamil	FA	Mixture of protein and ligand is injected	[181]
HSA	(±)-Benzoin, promethazine, thioridazine, propiomazine	ACE	P-BA	[169]
AGP	(+)- and (−)-benzoin, barbiturates, ifosfamide, cyclophosphamide, (*RS*)-disopyramide, beta-blockers	CEC	Immobilized AGP-stationary phase	[173]
Vancomycin	N-Acyl-D(L)-Ala-D(L)-Ala ligands	ACE	P-BA	[239,240]
Heat shock proteins (Hsp70 class)	Deoxyspergualin	ACE	Ligand in the running buffer, protein injected	[241]
Bovine carbonic anhydrase B	Arylsulfonamides	ACE	Ligand used as a buffer additive, protein injected	[174,175]
	Arylsulfonamides	CAGE	Affinity ligand immobilized in multisectional slab gel	[171]
C-Reactive protein	Ca^{2+}, phosphorylcholine	ACE	P-BA	[167]

ACE, affinity capillary electrophoresis; P-BA, protein used as a buffer additive; HDM, Hummel and Dreyer method; VPM, vacancy peak method; FA, frontal analysis; CAGE, capillary affinity gel electrophoresis; CEC, packed-capillary electrochromatography; BSA, bovine serum albumin; HSA, human serum albumin; AGP, human α_1-acid glycoprotein.

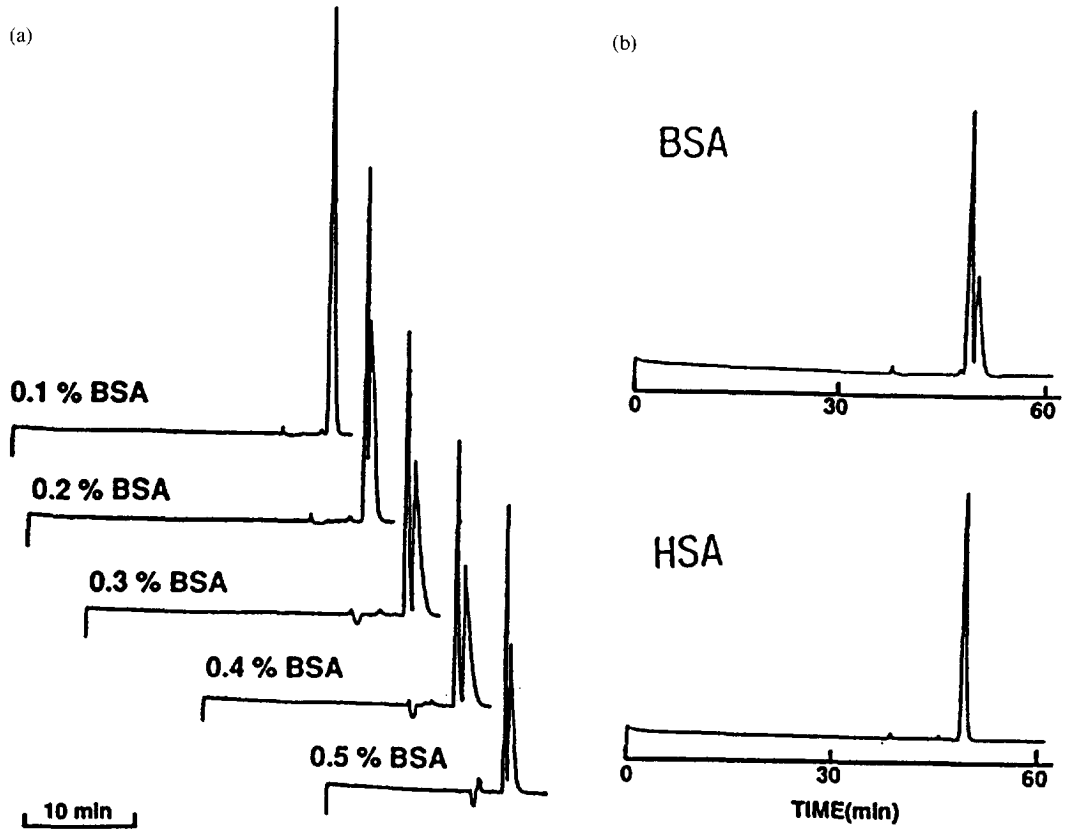

Fig. 5. (a) Electropherograms of (±)-ofloxacin chiral discrimination by using BSA as a running buffer additive. Analytical conditions: 75 μm I.D. capillary (effective length 35 cm), BSA diluted in 0.1 mol/l phosphate buffer (pH=7.0), 10 kV, 0.5 s hydrodynamic injection, detection: UV at 300 nm, temperature: 40°C. Reprinted, with permission, from Arai et al. [177]. (b) Comparison of (±)-ofloxacin chiral discrimination using BSA and HSA as chiral selectors. Analytical conditions: 50 μm I.D. capillary (effective length 45 cm), 0.3% protein solution in 0.1 mol/l phosphate buffer (pH=7.0), 10 kV, 1 s hydrodynamic injection, detection: UV at 300 nm, ambient temperature. Reprinted, with permission from Arai et al. [177].

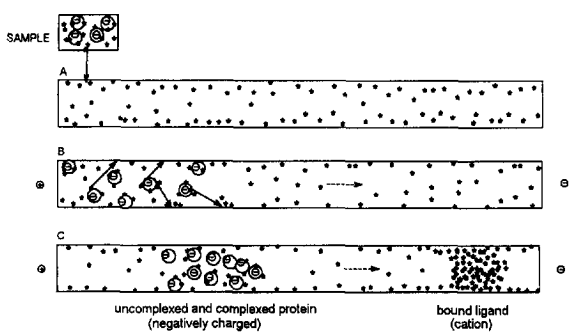

Fig. 6. Schematic presentation of the Hummel–Dreyer method by injecting excess of drug in the sample as compared to drug concentration in the mobile phase in capillary electrophoresis conditions. (A) establishing of the "first equilibrium" at the beginning of the sample injection; (B) "second equilibrium" (C) separation of drug–protein complex and "bound" drug.

ofloxacin–HSA interaction according to the slight differences existing in their primary, steric structures [176] (Fig. 5a and Fig. 5b).

Besides, this method also enables the evaluation of competitive drug–enantiomer (or enantiomer–enantiomer) interactions [169,177] and, by relating the electrophoretic mobility to binding, it is possible to determine ligand–protein binding parameters [169,178]:

$$\mu = \mu_f \frac{[L]}{[L] + [LP]} + \mu_p \frac{[LP]}{[L] + [LP]} \quad (4)$$

where μ is the overall mobility of the analyte, μ_f is the mobility of the free analyte, μ_p is the mobility of the analyte–protein complex, and [L] and [LP] are

the concentrations of the free and complexed ligand, respectively. By substituting $K_a = [LP]/[L][P]$, the equation transforms to:

$$\mu = \frac{\mu_f + \mu_p K_a[P]}{1 + K_a[P]} \quad (5)$$

where K_a is the equilibrium association constant and [P] is the free protein concentration. However, as reported [169], the determination of binding parameters by using this approach may become complicated due to presence of zone-broadening processes or exact temperature control. Special attention should also be paid to protein-wall adsorption and an appropriate (reproducible), capillary coating is often necessary.

Of course, in the same way as in affinity chromatography, the proteins in ACE may be immobilized in different ways (various gels, polymer networks, etc.) and ACE may be used as a rapid screening method of ligand–protein interactions as well as for the evaluation of ligand–ligand interactions at various protein-binding sites [170,171,173,179].

Recently, frontal analysis, the Hummel–Dreyer method (Fig. 6) and the vacancy peak method have been adapted by Kraak et al. [180] to capillary electrophoresis conditions. Interestingly, as showed by Shibukawa and coworkers by using frontal analysis (HPCE–FA), non-stereoselective [160], as well as stereoselective [181] aspects of HSA–verapamil interactions were described. The binding parameters obtained for (*RS*)-verapamil correlated very well with those obtained by equilibrium dialysis [160]. As compared to HPLC–FA, the sample volume in HPCE–FA is small (approx. 80 μl) and the separation is based on the measurement of differences in electrophoretic mobility of binding components. In this way it is possible to evaluate also interactions between molecules of similar size (but different electrophoretic mobility). The current major disadvantage of HPCE–FA is the relatively high detection limit and thus,the sensitivity is insufficient for the analysis of clinical samples (nanomolar range).

Briefly summarizing, in the future CE may be successfully used for drug–protein binding measurements, because all the interacting components can be studied in solution thus eliminating the possibility of denaturation or conformational alteration of the protein molecule. Simultaneous measurement of binding constants of multiple ligand interactions with protein is possible, only small (nanogram) quantities of protein and ligand are required, binding measurements can be carried out with mixtures of proteins and also with complex samples. Athough CE does not require the use of radioactive or chromophoric ligands, for an acceptable analysis time, it requires that the ligand (or running buffer modifier) is charged. Limitations of this method also include the need to minimize protein-wall adsorption and the need to increase the detection sensitivity for the measurements at therapeutic drug concentrations significantly.

3.4. Spectroscopy

Despite reports from some authors of a good correlation between binding parameters obtained by separation methods as compared to spectroscopic methods (and particularly fluorescence measurements, [182–184]), this approach is successful mainly for high-affinity binding sites and is poorly sensitive to low affinity interactions. Nevertheless, these methods facilitate insight into three-dimensional protein structure and thus elucidate some complementary structural and/or conformational variations of a protein molecule resulting from ligand attachment.

From the different spectroscopic methods (ultraviolet, visible, fluorescence, NMR) fluorescence spectroscopy has been used most widely. On the one hand, in studies considering mainly competitive binding aspects, a number of specific fluorescence markers have been employed either for albumin (dansylamide for site I; dansylsarcosine and dansylglycine for site II [185]) or for AAG (Auramine O [182], CDBA: 7-chloro-2-(*p*-diethylaminophenyl)-2*H*-benzotriazolyl-5-amine [186] and ANS: 1-anilino-8-naphthalene sulfonate [187]). On the other hand, by estimating the quantitative binding parameters of a fluorescent drug, experiments were based on measurement of the intrinsic molar fluorescence (ψ_B) of the protein-bound drug. Since this cannot be done directly (protein-bound drug should be followed under equilibrium conditions, i.e. without the separation of protein-bound and free drug fraction), it is a common praxis to measure fluorescence at constant ligand concentration and increasing protein concentrations. Extrapo-

lation of the fluorescence intensity against protein concentration in a double reciprocal plot enables to calculate the value of the intrinsic molar fluorescence of bound ligand (F_b). But theoretical analyses of the relationship between fluorescence intensity and protein concentration have revealed that these double-reciprocal plots exhibited nonlinearity, i.e. the value of F_b cannot be determined by simple linear extrapolation of the data [188,189]. An alternative graphical method in order to estimate accurately the F_b-value uses either direct plotting of emitted fluorescence intensity against the logarithm of the binding protein concentration [190], or simultaneous analysis of results at constant protein concentration and at constant ligand concentration by using non-linear least squares regression analysis of the experimental data [191]. Another shortcoming of the above mentioned approach (dependency of binding affinity on protein concentration) will be discussed in Section 5 of this review.

From other spectroscopic methods which have been used to characterize the interactions of macromolecules with small ligands, proton (^{1}H) nuclear magnetic resonance spectroscopy should be mentioned. However, in order to obtain reproducible and reliable spectra of subtle alterations in protein structure after ligand complexation, high field NMR spectrometers with advanced resolution and computer technology for data processing are required. The interpretation of results is based on the fact that drugs interacting with protein in a nonspecific (low-affinity) manner show different spectra, often with only somewhat broadened but not resolved signals of drug molecules. In contrast, the more specifically bound drugs cause usually a definite structural change of the "surrounding" protein molecule (and/or the structural changes facilitate the high-affinity binding), and consequently, in NMR difference spectra the drug signals are either very broadened, shifted or cannot be determined at the positions where the free drug signals were observed [192]. For special purposes, ^{19}F NMR spectroscopy has been successfully used to monitor the interactions of fluorinated drugs (5-fluorotryptophan, 5-fluorosalicylic acid, flurbiprofen or sulindac) with HSA [193]. It is of importance that the signals derived by NMR spectroscopy not only indicate the location of binding site, but also provide information on the residues of protein involved in the drug binding.

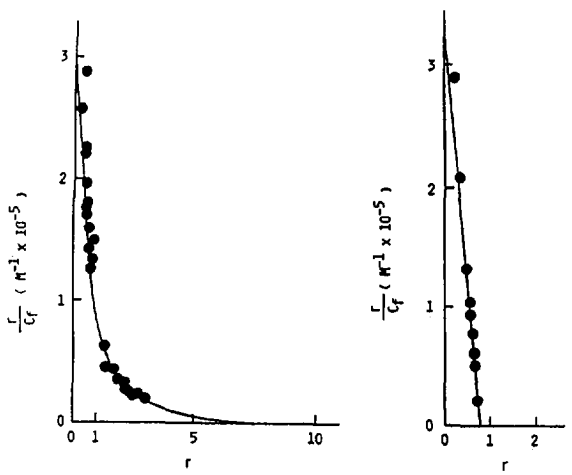

Fig. 7. Comparison of binding data obtained for (±)-pirprofen–HSA interaction studied by equilibrium dialysis (37°C, left panel) and circular dichroic method (25°C, right panel); HSA concentration: 50 μmol/l, pH=7.4 (0.067 mol/l phosphate buffer). The dialysis data were analyzed assuming two independent classes of binding sites: $n_1 = 0.9$, $K_1 = 3.9 \times 10^5$ l/mol, $n_2 = 2.9$, $K_2 = 8 \times 10^3$ l/mol; the line for CD data was calculated using $n_1 = 0.8$, $K_1 = 4.3 \times 10^5$ l/mol. Reproduced, with permission, from Otagiri et al. [206].

Current developments in high field (500–600 MHz) NMR technology offer a lot of promising opportunities for detailed mapping of binding interactions including their stoichiometry, kinetics and confomational properties of complexes, due to very high resolution of NMR signals [194,195]. The study of enantiomeric binding differences, when stereoselective ligand–protein interactions are considered, may be another challenging task for this approach in the near future. Predominantly, however, the mapping of

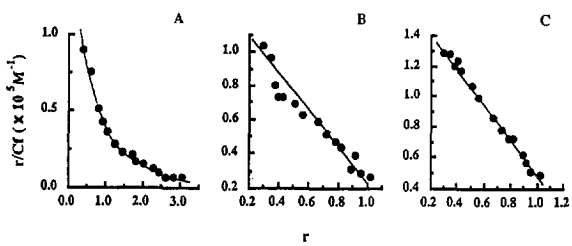

Fig. 8. Comparison of binding data obtained for (±)-suprofen–HSA interaction determined by equilibrium dialysis (A), circular dichroism (B) and fluorescence quenching (C) at pH=7.4 and 25°C. Calculated binding parameters: (A) $n_1 = 1.05$, $K_1 = 1.40 \times 10^5$ l/mol, $n_2 = 2.9$, $K_2 = 3.76 \times 10^5$ l/mol; (B) $n_1 = 1.09$, $K_1 = 1.50 \times 10^5$ l/mol, (C) $n_1 = 1.12$, $K_1 = 1.00 \times 10^5$ l/mol. Reproduced, with permission, from Maruyama et al. [208].

primary (high-affinity) binding sites is usually achieved by using NMR spectroscopy. Monitoring of the additional presence of secondary low-affinity binding sites does not seem possible [196] and/or has not been described yet. A further limitation of this method is the use of drug concentrations that are not pharmacologically relevant (typically in the millimolar range).

Chiroptical methods, optical rotatory dispersion (ORD) or circular dichroism (CD), have been proposed as a useful tool to monitor dynamic movements of protein conformation (and the role of relevant protein functionalities) in order to obtain a more complex information on the binding mechanism involved [197–199]. The conformational status of a protein and modification of its secondary and tertiary structure due to complexation with a particular drug is of crucial importance for binding properties of the relevant binding sites. This has been demonstrated for proton-linked conformation changes of albumin in the physiological pH range 7.0–9.0, with the N-form occurring below neutral pH and the B-form at higher pH (the N–B transition) [200–203]. Consequently, in non-stereoselective assays it has been shown that ligands are bound differently to the N and B forms of HSA: warfarin and diazepam exhibited higher affinity for the B-form [201,204,205], pirprofen and benoxaprofen were bound preferentially to the N form [206,207], whereas suprofen affinity does not seem to be affected by N–B transition [208]. Evaluation of possible conformational alterations of acceptor molecules are of major significance for the description of binding mechanism of stereoselective interactions and should be considered in the experimental study design. According the method of Rosen [209] the drawing of a tangent to the plot of induced ellipticity against the drug/protein ratio allows the estimation of the free and bound fractions of a drug and the calculation of the binding parameters.

However, in more detailed binding studies evaluating both qualitative and quantitative aspects of drug–protein interactions by employing a variety of complementary experimental methods (equilibrium dialysis, UV absorption, fluorescence, CD and ^{1}H NMR), a clear discrepancy between results acquired from CD vs. equilibrium dialysis has been observed as related to the binding mechanism (Fig. 7 and Fig. 8), i.e., a binding model assuming one vs. two independent classes of binding sites has been considered for CD-data and dialysis data, respectively. Although the binding parameters (n, K_a) of high-affinity binding sites derived by the CD method were in reasonable agreement with those obtained by the dialysis method [206,208], Otagiri and coworkers have provided clear evidence that only the presence of primary high-affinity binding sites could be identified by using spectroscopic techniques (CD or fluorescence quenching method) as compared to equilibrium dialysis. Maruyama et al. [208] have ascribed this inconsistency to the fact that drug molecules are "optically inactive" at the second class of binding sites. Monitoring of heterogeneous binding processes (concomitant presence of low-affinity binding component(s)) is usually not possible by CD and according to this, quantitative information derived from these studies should be interpreted carefully.

3.5. Other methods

Rather exceptionally, some other methods have been used for drug–protein binding studies, with respect to unique features of the ligand or to reveal specific qualitative or quantitative aspects of interaction. Examples include the use of polarography [210], calorimetry [137,211] or stopped-flow experiments for evaluation of binding kinetics [56]. Stopped-flow analysis was able to reveal different drug binding kinetics at site II for abumin in diabetics. The fluorescence polarization immunoassay (FPIA) method has been introduced for therapeutic drug monitoring (Abbott TDX System) and for free concentration of some drugs (disopyramide), a very good correlation between FPIA and the reference method has been described [212].

A "physiological approach" of membrane separation experiments in vitro is represented by red blood cell uptake studies [213,214] employing the erythrocytic membrane as a diffusional transfer barrier: this approach may be useful for lipophilic drugs to prevent their adsorption to the dialysis membrane or apparatus.

Alternatively, although not described in literature yet, the analytical potential and applicability of field-flow fractionation (FFF) for characterization of

binding interactions should be mentioned [215,216]. The sedimentation FFF is a technique measuring effective mass/mass distribution of particles (and it is sensitive to even small changes of it) as compared to flow FFF providing a direct measurement of particle size/size distribution, or gravitational FFF operating using the earth's gravitational field. In summary, component properties measurable by FFF include mass, size, density, charge, diffusivity and sample range include a broad macromolecular-colloidal-particulate spectrum (1 nm–100 μm). The use of raw, complex samples (protein mixtures, aggregates, cells, etc.) increases further the attractiveness of this flexible technique for drug–protein binding assays.

4. Monitoring of free drug in vivo

Sampling and determining of "true" non-protein bound fraction of a drug in dynamically functioning living biological system is of utmost pharmacological importance. However, it suffers from several methodological problems. Obviously, a complex approach should reflect the existing organ differences as well as the "free intermediate hypothesis", i.e. the fact that a part of the initially bound drug is eventually released, depending on capillary transit time, the rate of dissociation of the drug–protein complex and the permeability and surface area of the capillaries [6,217,218]. Although measurements of saliva or cerebrospinal fluid has been proved to estimate the free drug concentration at target receptor sites, they are only of limited general utility for free drug therapeutic monitoring [2,219].

The use of microdialysis perfusion technique was proposed as an in vivo alternative for the study of the steady-state free fraction of drug in different tissues and body compartments [19,20,220,221]. In microdialysis sampling, a hydrophilic capillary (small-diameter dialysis tubing) is implanted in biological compartment(s) of particular interest. By perfusion of the capillary with a physiological solution at a low flow-rate (usually less than 2 μl/min), low molecular weight compounds diffuse easily through the membrane into the perfusate in contrast to proteins, or other higher molecular weight compounds, which do not enter the perfusion medium. In summary, microdialysis offers advantages in terms of maintaining equilibria and experimental versatility in vivo (time-dependent sampling, rapid continuous sampling avoiding enzymatic degradation of the sample, or sampling in awake, freely moving animals).

The alternative use of this method for in vitro plasma protein binding studies has been studied [222] and further evaluated in a modified dynamic system (artificial blood vessel) for determination of the free concentration of drugs (paracetamol, procainamid, caffeine, theophylline, lidocaine, carabamazepine, phenobarbital and phenytoin) in vitro [223]. The results validated microdialysis as a method which operates by preserving binding equilibria over a wide range of drug concentration and wide range of protein binding. Since it is possible to choose from a variety of probe types with different MWCO-membranes and also to implant the microdialysis probes intravenously [224], the extent of drug binding to proteins (and other plasma components) could be studied continuously in vivo under physiological conditions. Owing to the relatively small volumes of samples (20–50 μl: sample collection rate is usually 0.1–10 μl/min and typical time resolution 1–5 min), the major limitation of this promising method is the lack of a currently available analytical method with sufficient sensitivity.

Capillary ultrafiltration, employing an active-pressure gradient instead of a passive diffusion concentration gradient for the sampling process, is another new technique with the ability to monitor unbound drug in living biological systems in vivo [225].

5. Evaluation of binding data

The usual approach of ligand binding studies is to fit the experimental data to the Eq. 3 (Section 2.1 and to plot them in form of a so-called Scatchard plot [21] (Fig. 7 and Fig. 8). The most important limitations concerning this approach have been rewieved recently [111,226–229] and include either the oversimplification of ligand attachment to the binding site(s) by fitting of curvilinear plots with straight lines or contrarily, the detection of visionary,

biochemically or pharmacologically not interpretable acceptor heterogeneity. At least in part, a number of experimental artifacts depending on the methodology applied may explain the curvilinear nature of Scatchard plots: the reasons for downward curvature are represented predominantly by incomplete recovery of bound fraction, irreversible ligand binding or its internalization, ligand or acceptor degradation as well as non-equilibrium binding conditions; the upward curvature is usually caused by affinity difference between labeled and unlabeled ligands, imprecise estimation of non-specific binding or by contamination of the bound fraction with unbound ligand [229]. Alternatively, some factors (impurities in labeled ligand preparation or influence of membrane microenvironment) may result in either upward or downward curvature of Scatchard plots. As it is clearly demonstrated by Fig. 7 and Fig. 8, some low-affinity binding components may be overlooked by incorrect data analysis. In addition, other obvious reasons for variability in binding parameters are that a ligand's association constant and/or the number of binding sites increase when the acceptor (receptor or protein) preparation is diluted [111]. This point is related partly to the very common situation when a competitive inhibitor (or contaminant) of binding is present in the acceptor preparation. In such a case Eq. 3 could be transformed to the form:

$$B = \frac{n_i K_{a_i} F}{1 + K_{a_i} F + K_i I} \quad (6)$$

where I is the free molar concentration of inhibitor and K_i is the corresponding association constant. When a competitive inhibitor is present at a fixed concentration ratio to the acceptor, a strong similarity of Scatchard plot to the binding model described by Eq. 3: $z > 1$ (i.e. curvature of Scatchard plot) could be observed. Obviously, all the above mentioned aspects are plausible also for the evaluation of stereospecific drug–protein interactions (inclusively enantiomeric contamination) [154,158,230].

Generally, although the Scatchard plot will probably be further used for illustration and comparison of different binding data sets, for quantitative evaluation purposes some alternative graphical representations (particularly the Bjerrum's plot) have been considered. The major advantage of them (discussed in more detail by Klotz [226,227]) is the direct representation of experimental data. As pointed out by Kermode [229] the application of linear regression to transformed (Scatchard) data in order to calculate the binding parameters could no longer be accepted as an appropriate quantitative approach. The graphical and statistical analysis of raw, untransformed data has become an imperative in ligand–protein binding studies.

6. Clinical and therapeutic relevance of drug–protein binding studies

The pharmacokinetic and clinical consequences of drug binding to proteins are usually discussed in terms of possible binding displacement interactions [11–13]. Accordingly, some algorithms for guessing the clinical significance of such interactions have been suggested. It seems likely that protein-binding displacement phenomena may be clinically significant only for highly protein-bound (>90%), low clearance drugs with a narrow therapeutic index and a small distribution volume administered intravenously (examples include warfarin, phenytoin or tolbutamide, an exception to the rule is represented by lidocaine [13]).

From a methodological point of view (and also taking into account stereoselective displacement possibilities), there will be always a need for a relatively simple and effective indicative screening method for the evaluation of potential displacement aspects caused by the development and introducion of new, particularly chiral drugs. The recent status and future perspectives of some of the analytical approaches mentioned, for example the use of binding proteins as PSPs in affinity chromatography (Section 3.2.1) or as running buffer additives in CE (Section 3.3), offer promising and powerful tools.

7. Concluding remarks

It has been shown that the development of new analytical and experimental methodologies impact remarkably on the knowledge and understanding of complex mechanisms involved in the process of drug–ligand binding to different protein-binding

sites. Obviously, only by the adoption of an appropriate (i.e. sensitive and specific) methodological approach is it possible to evaluate the sometimes very subtle and dynamic changes at the relevant protein-binding sites, particularly in the presence of drug enantiomers, structural derivatives or unstable intermediates of drug metabolism (such as acyl glucuronides). This holds true also for stereoselective and nonstereoselective competitive binding studies, species-dependent protein-binding differences as well as for the genetically, metabolically or pathophysiologically altered protein-binding profile of a particular protein. For example, the description of differences in binding properties which exist between the genetic variants of AAG has provided recently a plausible explanation why some authors reported a small or non-integral number (n between 0.2 and 0.7) of high affinity sites for the binding of basic and acidic drugs to AAG [18]. During the past decade, impressive progress in stereospecific assays has been achieved, facilitating more exact knowledge of mechanisms responsible for enantiomeric binding discrepancies. It is most important to take these differences into consideration when analyzing interactions of chiral drugs with proteins. On the other hand, if stereoselective aspects are neglected when results of many studies are being extracted and interpreted, their relative plausibility should be outlined.

Although conventional methods like equilibrium dialysis or ultrafiltration are at present widely applied for rapid screening in preclinical and clinical drug–protein binding studies, for many drugs (especially for hydrophobic, highly protein-bound ligands) the use of an alternative methodology (chromatography, capillary electrophoresis) has been recently validated [160–164] and it is therefore highly recommended. As it was demonstrated [163,231], these methods also offer, besides convenient experimental design (small quantities of protein and ligand required, speed and high efficiency due to the application of automatic systems), the possibility of studying the (stereoselective) drug interactions with complex biological matrices (plasma, serum). By introducing biocompatible (ISRP) type HPLC columns [149,181], the one potential disadvantage of the chromatographic approach (related to the possible alteration of protein conformation which resulted from its interaction with stationary phase) seems to be effectively minimized. By using capillary electrophoresis methods, the major limiting factor is the not sufficiently sensitive detection, particularly for measurements at "therapeutic" (nanomolar or femtomolar) drug concentrations. Nevertheless, the continuous introduction of sophisticated technical improvements (various laser-based detection systems) makes us optimistic about future progress.

The "optimal" experimental strategy of in vitro binding studies should take into account also the structural or conformational status of binding sites, with respect to its alteration as a consequence of ligand binding at different drug/protein molar ratios. Therefore, complementary information from spectroscopic (CD, fluorescence or ^{1}H NMR) measurements are often very useful, providing essentially new insight into the actual binding mechanism(s) involved. However, as revealed in some comparative studies using different analytical methods, this approach is not suitable for the extraction of quantitative binding parameters, particularly in the case of "heterogeneous" binding processes (i.e. when the ligand interacts with more than one class of binding sites with different binding affinity).

In general, for an objective analysis of protein-binding behaviour of a particular drug in physiological and/or pathophysiological status, it is important and advantageous to know also complementary information from in vivo binding experiments. For example, the metabolic incorporation of highly lipophilic drugs (cyclosporin) into lipoprotein structural components taking place in vivo cannot be adequately simulated in in vitro conditions [232]. Because of its experimental versatility, the microdialysis technique offers at present the most promising preclinical methodological alternative for monitoring of dynamic changes of free drug in vivo in different body compartments. Samples from the microdialysis probes are suitable for HPLC (or CE) analysis and systems and direct on-line connection between microdialysis and HPLC apparatus has been already successfully applied [224].

Finally, as mentioned briefly in Section 5 of this review, some methodological shortcomings and artifacts may cause significant changes in resulting binding data sets (e.g. curvilinear nature of Scatchard plots). Therefore, the data analysis procedure should

also take into account the potential interference of experimental conditions.

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