CHAPTER 10

Protein binding in plasma: a case history of a highly protein-bound drug

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10.1 *Introduction*

Protein binding is commonly defined as the reversible association of a drug to the proteins of blood, or more usually plasma. When a drug is administered intravenously or reaches the blood circulation after absorption, it interacts with the available proteins in two different ways: by adsorption on the surface of the proteins or, more rarely, by covalent bonding with active chemical groups of the proteins. When the binding is reversible, there is a dynamic distribution with a transfer between proteins and plasma water. At equilibrium a certain fraction of the total drug amount is bound to plasma proteins. Any factors modifying the nature of the binding interactions (pH change, ionic strength, protein tertiary structure, temperature) and the presence of other competing ligands can modify the extent of binding.

Investigation into the protein binding of new chemical entities is an important activity in the drug development process due to its role in determining clearance and distribution parameters. A summary of the basic concepts with some technical considerations is given, together with a description of a case history of a highly bound drug.

10.2 *The protein binding equilibrium*

The interaction of drugs with proteins can be treated as an equilibrium, obeying the law of mass action kinetics. Therefore it can be described by the reversible equation:

$$[\mathrm{D}] + [\mathrm{P}] \Leftrightarrow [\mathrm{DP}] \tag{10.1}$$

where [D], [P] and [DP] are the molar concentrations of unbound drug, unoccupied protein binding sites and drug–protein complex, respectively, and k_{on} and k_{off} are the rate constants for the forward (association) and reverse (dissociation) reactions, respectively. The equilibrium *association constant* (K_a, molar^{-1} units) for this reaction is defined as:

$$K_a = \frac{k_{on}}{k_{off}} = \frac{[\mathrm{DP}]}{[\mathrm{D}][\mathrm{P}]} \tag{10.2}$$

and it provides an index of the affinity between the drug and the binding sites. The total concentration of the binding sites is the sum of unoccupied [P] and occupied binding sites [DP]: this total concentration of sites is referred to as the *capacity constant* (N) and has units of sites/L. Since a given protein can have several classes of independent binding sites, the capacity constant represents the product of the 'number of sites/mole of protein' and the molar concentration of protein. Combining all these equations and considering all classes of binding sites, the concentration of bound drug [DP] in a protein solution can be expressed as:

$$[\mathrm{DP}] = \sum \frac{Ntot_i[\mathrm{D}]}{Kd_i + [\mathrm{D}]} \tag{10.3}$$

where i refers to the number of different classes of binding sites.

10.3 *Determinants of the unbound fraction*

Equation 10.3 can be rearranged so that the *unbound plasma fraction* (fu) of drug is represented as:

$$fu = \frac{[\mathrm{D}]}{[\mathrm{DP}]} = \frac{Kd + [\mathrm{D}]}{Ntot + [\mathrm{D}] + Kd} \tag{10.4}$$

Therefore, for a drug that binds to a single class of binding site, the bound drug fraction depends on the equilibrium-free drug concentration, the dissociation constant and the protein concentration. Normally the fraction bound remains constant within a wide concentration range, including the therapeutic range. However, for some drugs (e.g. valproic acid, salicylic acid and several non-steroidal anti-inflammatory drugs), proteins appear to have very limited binding capacity, showing an increase of unbound drug when the total drug concentration is increased (concentration-dependent binding (Lin, 1987; MacKichan, 1992)). Plasma proteins in general have a high capacity for binding and are able to 'extract' certain drug molecules from aqueous solution or suspension. This can be due to a large number of binding sites on each protein molecule and/or high binding affinity. Specific binding sites (high K_a values, low binding capacity) predominate at low drug concentrations, while non-specific binding ones (low K_a values, high binding capacity) predominate at higher concentrations. The possibility of a cooperative binding has also been suggested whereby the affinity of the drug increases as more binding occurs.

10.4 *Principal plasma binding proteins*

Human plasma contains about 100 proteins of which 13 are present at concentrations higher than 1 g/L. Among these latter, six are able to bind drugs: human serum albumin (HSA), α1-acid glycoprotein (AAG), lipoproteins (VLDL, LDL, HDL) and immunoglobulins G (IgG) (Table 10.1). However, albumin is by far the largest contributor to plasma protein binding since it represents 60 per cent of the total plasma proteins. It is principally involved in the binding of most anionic drugs and many endogenous anions. However, many cationic and neutral drugs also bind appreciably to AAG and/or lipoproteins (Table 10.2).

Various disease states can modify the extent of the binding of drugs to plasma proteins (Tillement *et al*., 1978; MacKichan, 1992). The modification can be due

TABLE 10.1 *Characteristic of the drug binding proteins in plasma*

	Albumin	**AAG**	**Lipoproteins**
Molecular weight	66300	40000	200000–1000000
Normal serum conc. (mg/100 mL)	3500–5500	55–140	VLDL: <40 HDL: >35 LDL: 70–205
Half-life (days)	19	5.5	up to 6
Distribution	40% intravascular 60% extravascular		

TABLE 10.2 *Predominant binding proteins of some common drugs bound >70 per cent to plasma proteins*

Albumin	Albumin and AAG	Albumin and lipoproteins	Albumin, AAG, and lipoproteins
Ceftriaxone (A)	Alprenolol (B)	Cyclosporine (N)[b]	Amitriptylline (B)
Clindamycin (A)	Carbamazepine (N)	Probucol (N)[b]	Bupivicaine (B)
Clofibrate (A)	Disopyramide (B)[b]		Chlorpromazine (B)
Dexamethasone (N)	Erythromycin (B)		Diltiazem (B)
Diazepam (B)	Lidocaine (B)		Imipramine (B)
Diazoxide (A)	Meperidine (B)		Nortriptyline (B)
Dicloxacillin (A)	Methadone (B)		Perazine (B)
Digitoxin (N)	Verapamil (B)		Propranolol (B)
Etoposide (N)			Quinidine (B)
Ibuprofen (A)			
Indomethacin (A)			
Nafcillin (A)			
Naproxen (A)			
Oxacillin (A)			
Phenylbutazone (A)			
Phenytoin (A)			
Probenecid (A)			
Salicylic acid (A)			
Sulfisoxazole (A)			
Teniposide (N)			
Thiopental (A)			
Tolbutamide (A)			
Valproic acid (A)			
Warfarin (A)			

[a] A = Acid; B = Base; N = Neutral.
[b] Albumin is minor binding protein.

to a series of both physiological (pregnancy, gender, smoking, obesity, nutritional status, surgery, extremes of age) and pathological conditions (renal and liver disease, infarction, cancer, injuries, diabetes, thyroid diseases, cystic fibrosis, inflammatory arthritis, hyperlipoproteinemia).

10.4.1 HUMAN SERUM ALBUMIN (HSA)

Albumin is a single peptide chain of about 580 amino acid residues. The primary physiological roles of albumin are to maintain colloid osmotic pressure in the vascular system and to transport fatty acid and bilirubin. It is not confined to

plasma, but is continuously filtered at a slow rate into interstitial fluid. It is also present in the cerebrospinal fluid (Table 10.3). Albumin-bound drug is therefore found not only in plasma, but also in the interstitial fluid, which contains 60 per cent of the albumin in the body.

Two primary areas of 'high affinity' drug binding sites have been defined on albumin. These have been nominated the *warfarin site* (site I) and the *benzodiazepine site* (site II). Both the sites are in reality shared with other drug and endogenous compounds. Several drugs (e.g. naproxen, tolbutamide, indomethacin) bind to both sites. With the help of fluorescent probes, additional binding sites and their locations have been proposed (Hervé, 1994) (Figure 10.1). Basic drugs can also bind to albumin, but binding of such drugs in plasma is too high to be accounted for by their binding to albumin alone. Albumin tends to show a low affinity and a high capacity for these basic drugs and a variation in albumin concentrations, for example, does not result in marked changes in their plasma protein binding.

10.4.2 α1-Acid glycoprotein (AAG)

AAG (orosomucoid) is a glycoprotein with a lower molecular weight than albumin and is characterised by its high carbohydrate content. It is a monomer of 181 amino acids and 40 per cent of glucid residues. Binding of drugs to AAG appears to involve hydrophobic rather than electrostatic forces. AAG is present in plasma at concentrations which normally are 100 times lower than for albumin. In contrast to the homogeneity of albumin, polymorphic forms of AAG are normally found (Lunde, 1986). These variants may be selective and possess different drug binding capacities. The drug binding sites are believed to be located on the polypeptide chain and may be shared by both acids and bases. Although AAG is known to be a major binding protein for many basic drugs, it also binds some acidic and neutral drugs, but to a lesser extent. Warfarin, for example, can compete with drugs that bind to AAG. In general, AAG is referred to as a 'low capacity, high affinity' protein, while albumin is a 'high capacity, low affinity' one. The plasma concentration of AAG is very much subject to disease and stress state. Elevated levels of AAG are seen in different states of inflammation or injury, therefore an increase in AAG-binding capacity can be observed in these patients compared to healthy volunteers.

Table 10.3 *Normal albumin content in various fluids*

	Plasma	Transudates	Pleural fluid	Pericardial fluid	Peritoneal fluid	CSF
Albumin (g/L)	40	20	9	20	9	0.2

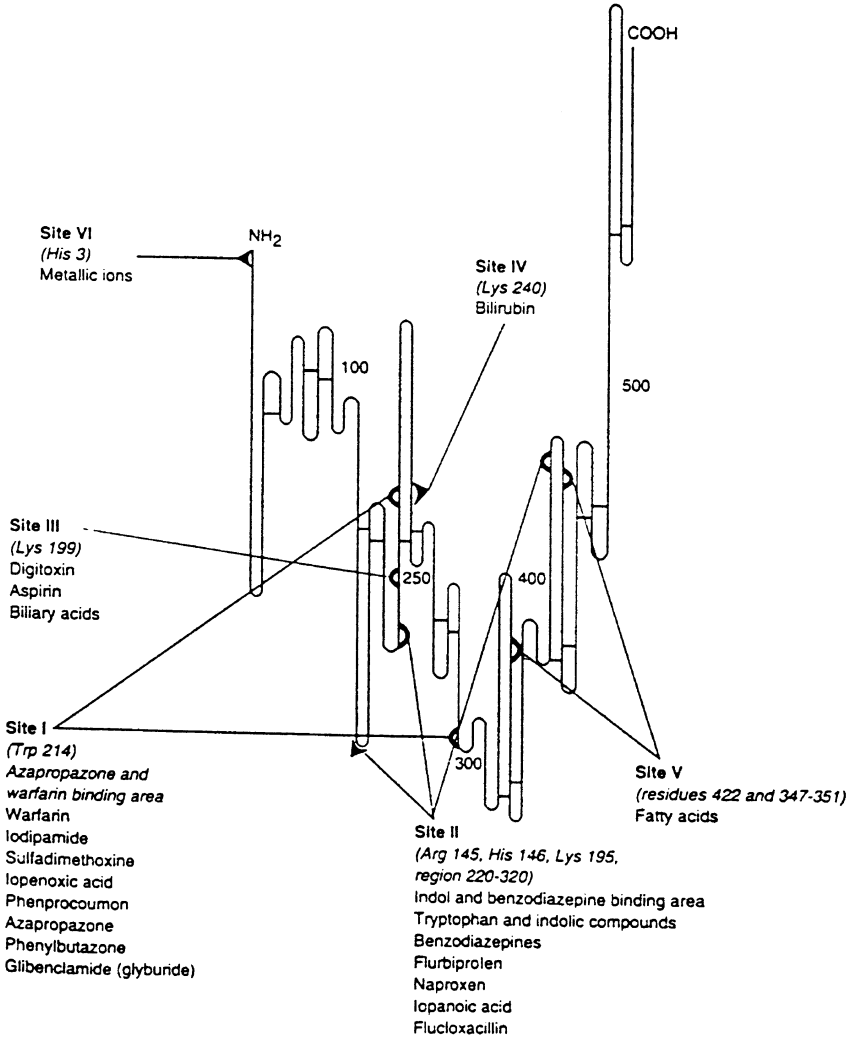

FIGURE 10.1 *Location of binding sites in human serum albumin* (reproduced with permission from Adis International).

10.4.3 LIPOPROTEINS

Lipoproteins are an extremely heterogeneous group of proteins that have a wide range of molecular weights and lipid contents (Zini, 1991). Their tertiary and quaternary structures are not fully elucidated but polar proteins and lipids surround a hydrophobic centre composed of non-polar lipids. The three most important groups are: very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL) (Table 10.4). Their concentrations can vary

TABLE 10.4 *Characteristic of plasma lipoproteins*

	VLDL	LDL	HDL
Molecular weight	10^7	3×10^6	3×10^5
Proteins (%)	10	20	50
Cholesterol (%)	20	48	10
Phospholipids (%)	20	24	22
Triglycerides (%)	50	8	18
Fasting conc. (g/L)	1.2	55	32.5
Plasma amount (%)	12.5	55	32.5

extensively in the normal population. They transport fatty acids, triglycerides, phospholipids and cholesterol and may also be responsible for the binding of certain drugs such as chlorpromazine, imipramine, probucol, cyclosporine, tetracyclines and nicardipine. However, nearly all types of drugs are capable of binding to isolated lipoproteins, provided they exhibit a certain degree of lipophilicity. Drugs that 'bind' to lipoproteins are thought to actually be partitioning into the lipid core of the protein instead of associating with a specific site. For this reason competition between drugs for specific sites is not likely to occur and concentration-dependent binding is not expected.

10.5 *The importance of protein binding in drug development*

10.5.1 PHARMACOKINETICS

Plasma protein binding can be important in determining drug distribution, metabolism and elimination. It is widely accepted that only the unbound drug can diffuse across membrane barriers and interact with metabolic enzymes. The interaction of the free drug with the tissues can also affect its distribution. The determinants of binding in tissues exposed to the drug are the same as those for plasma: protein concentration, affinity to the proteins, unbound drug concentration available. However, overall tissue binding in the body (the unbound fraction, fu_t) is difficult to measure, though most recent techniques (i.e. microdialysis) may help.

The PK parameters, which are the determinants of the drug concentration–time plasma profile, are steady-state volume of distribution (V_{ss}), clearance (CL) and elimination half-life ($t_{1/2}$). It is important to understand the influence of protein binding on each of these parameters.

Volume of distribution

The magnitude of drug binding in plasma versus that in tissue is the primary determinant of the apparent volume of distribution of a drug:

$$V_{ss} = V_p + \left(\frac{fu_p}{fu_t}\right) \times V_t \tag{10.5}$$

where fu_p is the unbound fraction in plasma, fu_t is the unbound fraction of drug in tissue, V_p is plasma volume (approx. 0.07 L/kg in humans), V_t is the tissue volume (0.6 L/kg in humans).

An estimate of overall tissue binding in the body can be made when V_{ss} and fu_p are known and anatomic volumes are assumed.

Many drugs (e.g. amiodarone, many tryciclic antidepressants) have very large distribution volumes (20–70 L/kg) because they are much more highly bound in tissue than to plasma proteins. On the other hand, the small distribution volumes of warfarin, valproic acid, penicillins (0.1–0.5 L/kg) are attributable to high plasma binding relative to tissue binding.

Clearance

The understanding of the relationship between protein binding in plasma and organ clearance is aided by the physiological model of hepatic clearance (Wilkinson and Shand, 1975). According to this model, an organ clearance, and hence the average steady-state concentration C_{ss}, is determined by three physiological variables: organ blood flow (Q), intrinsic organ clearance (CL_{int}) and the unbound fraction of drug in blood (fu_b) as follows:

$$CL = Q \times \frac{CL_{int} \times fu_b}{Q + CL_{int} \times fu_b} \tag{10.6}$$

The concept of 'restrictive' and 'non-restrictive' clearance needs to be adopted in order to explain how protein binding is involved in the clearance process. A drug is said to undergo restrictive clearance when intrinsic organ clearance is such that:

$$Q \gg CL_{int} \times fu_b$$

Thus, equation 10.6 can be simplified to:

$$CL \approx CL_{int} \times fu_b \tag{10.7}$$

According to this relationship, the clearance is proportional to the unbound fraction. Displacement of plasma protein binding of one drug by a co-administered drug is possible: however, as long as there is no effect on intrinsic organ clearance (enzyme induction/inhibition), effects of protein-binding displacement will not

affect the average free plasma concentration of drugs. Figure 10.2 shows the effect of an increase of unbound fraction (i.e. after displacement) on C_{ss} (unbound), C (bound), CL and fu.

In the case of non-restrictive clearance, protein binding does not appear to protect the drug from elimination. The clearance is determined primarily by organ blood flow, is less influenced by intrinsic clearance and is independent of the unbound fraction. To complicate the situation still further, there is a growing evidence that also the bound fraction, in the form of AAG or lipoprotein complexes, may be taken up into organs by endocytotic mechanisms.

Elimination half-life

The elimination phase is closely related to V_{ss} for most drugs. The half-life of a restrictively cleared drug which has a moderate-to-large V_{ss} value (i.e. >0.4 L/kg) is less influenced by V_p (10.5). It is determined by the simplified equation:

$$t_{1/2} \approx 0.693\left(\frac{V_t}{CLu_{int} \times fu_t}\right) \tag{10.8}$$

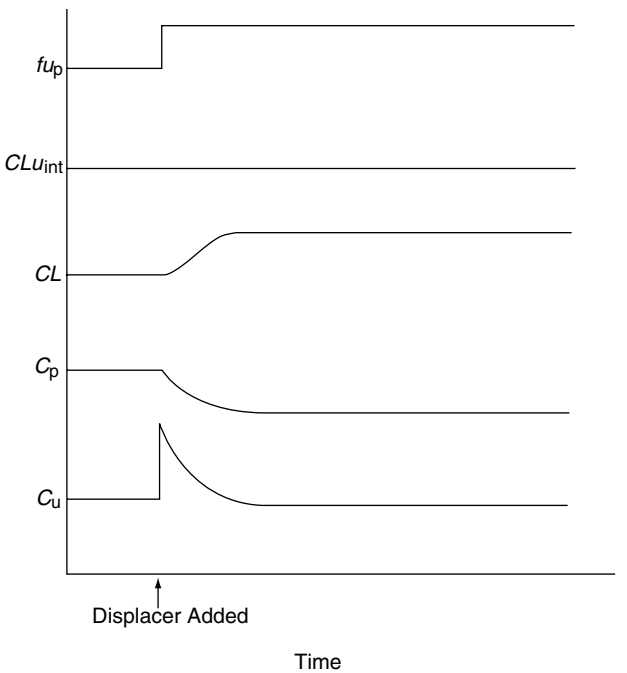

FIGURE 10.2 *Time course of changes in unbound drug fraction (fu_p), total (C_p) and unbound drug concentrations (Cu_p) for a displaced drug that is restrictively cleared and assuming no change in instrinsic clearance.*

So, altered plasma protein binding will have little effect on the half-lives of high distribution volume drugs such as diazepam and phenytoin. When V_{ss} values are less than 0.4 L/kg (warfarin), $t_{1/2}$ is affected by changes in plasma (and tissue) binding according to the simplified equation:

$$t_{1/2} \approx 0.693\left(\frac{V_p}{CLu_{int} \times fu_p} + \frac{V_t}{CLu_{int} \times fu_t}\right) \tag{10.9}$$

In the case of non-restrictively cleared drugs, the half-life is influenced by both plasma and tissue binding, according to:

$$t_{1/2} \approx 0.693\left[V_p + \left(\frac{fu_p}{fu_t}\right)V_t\right] \tag{10.10}$$

Decreased binding in plasma will increase the V_{ss} and prolong the half-life, while increased plasma binding will shorten half-life. For drugs with very small volumes of distribution (penicillins), the effects of altered plasma and tissue binding on elimination of half-life will be minimal.

10.5.2 Toxicological cover

Traditionally, in order to predict a potentially toxic dose in man, the exposure of the species to the drug is measured using the plasma area under the time–concentration curve (AUC). Ideally, the AUC observed at the highest dose in man should not exceed that giving no toxicological effect in animals, and normally it is preferred that there be a reasonable margin to account for potential differences in distribution and elimination of the drug (safety margin). In cases in which the drug shows high restrictive plasma protein binding and/or wide variability in the extent of binding between humans and non-clinical species, it can be more meaningful to determine safety margins on the basis of unbound drug.

10.5.3 Protein-binding displacement

Drug–drug interactions due to alteration of the plasma protein binding of a drug by another drug have been the subject of many debates in the literature. Many of the first examples of clinically significant drug–drug interactions thought to be due to alteration of plasma protein binding were in fact due to metabolic interactions (Rolan, 1994). It was believed by many clinicians that a displacement of protein binding was potentially of critical importance as the free concentration is widely believed to represent the pharmacologically active fraction as only the free concentration is able to pass through membrane barriers. However, a displacement

of the protein binding of a drug will only cause a transient increase in free drug concentration, as for most drugs of low to moderate clearance the increase in free concentration will also cause an increase in clearance as well as an increase in volume of distribution. Therefore total concentration will fall until the free concentration reaches previous equilibrium levels (see Figure 10.2). Consequently, the only effect of a protein-binding displacement is to decrease total concentration, increase %unbound but the free concentration remains constant. Therefore, if the pharmacological action depends on the free plasma concentration, no difference in drug activity should occur as long as the transient increase is not relevant. However, this is only partly true as volume of distribution also increases, although not to the same extent as the increase in clearance (MacKichan, 1989; Rolan, 1994; Sansom, 1995).

10.6 *Techniques for measurement: a brief review of the more popular techniques including advantages and disadvantages*

The *in vitro* measurement of drug binding to plasma proteins is used to calculate PK parameters and make predictions on the PK behaviour of the drug. The major concern in this extrapolation from *in vitro* to *in vivo* is the possibility that the binding values could be misleading because of procedure artefacts. The advantages and disadvantages of the three most common methods for plasma protein binding are discussed below. These techniques are useful also in studies where single proteins are used in order to determine the major contributing proteins, kinetics of binding and type of binding sites although this information may have little physiological or clinical relevance. Moreover, some of the listed techniques are also applicable to investigate the binding of the drug to tissue homogenates.

It must be underlined that very often the protein binding values obtained using different techniques can be significantly different due to the different technical aspects. Thus, an overall recommendation is difficult to make as it is important to take into account the final aim of the investigation and the intrinsic characteristics of the drug. The available techniques have been extensively reviewed by Oravcova (1996), Wright (1996) and Zini (1991).

10.6.1 Equilibrium dialysis

Equilibrium dialysis has been by far the more widely used method to study ligand–protein interactions. As a typical example of equilibrium dialysis, the solution of a high molecular weight compound (the protein) containing a ligand (the drug) is separated by a semi-permeable membrane with a known molecular weight cut-off from a buffer solution. After the equilibrium has been reached, the concentration of the free ligand is equal on both sides of the membrane. According to the Fick's law, the rate

of diffusion of the ligand depends on the surface area, the thickness of the membrane, the concentration range, the filling volume and the diffusion coefficient. The latter is a function of both the molecular weight, temperature and characteristic of the ligand. To meet the optimal requirements, some dialysing systems (Spectrum® cells, for example) have been specially designed. With these cells, the ratio between the membrane surface and the working volume is optimised. Plasma and buffer are placed in their respective reservoirs separated by the dialysis membrane. The system is then incubated at the desired temperature and allowed to reach equilibrium. Then, the fluid from each reservoir is removed and analysed for drug concentrations. The post-dialysis drug concentration in the buffer reservoir (C_u) will be in equilibrium with the free drug concentration in the plasma reservoir so that the percent of binding will be as follows:

$$\%\text{bound} = \frac{C_t - C_u}{C_t} \times 100 \tag{10.11}$$

where C_t is the drug concentration in the plasma reservoir after dialysis. There are numerous variables that must be controlled in equilibrium dialysis experiments in order to obtain accurate results: the incubation temperature and time for incubation, the non-specific binding to the apparatus or membrane, the solubility of the drug in the buffer, the radiochemical purity of the drug (Joshi, 1994), the pH at which the incubation is performed, the volume shift due to Gibbs-Donnan equilibrium (Lima, 1983; Bowers, 1984).

10.6.2 Ultrafiltration

This method of separating drug-bound from free drug is extremely popular because of the availability of a large number of commercial easy-to-use filtration devices (Amicon®, Millipore®, BioRad®). Ultrafiltration is mainly used under negative pressure by centrifugation at 1,000–2,000 g for 15–30 minutes. The principle of this separation procedure is that incubation between drug and protein is terminated by putting the incubate through a filtration membrane of known molecular weight, cut-off with the free drug passing through the filter. The free concentration will be constant in ultrafiltrate and retentate. However, the free concentration remains constant during filtration provided that the filtered volume does not exceed 40 per cent of the introduced total volume. The percentage of unbound is calculated by:

$$\%\text{unbound} = \frac{C_u}{C_t} \times 100 \tag{10.12}$$

where C_u is the drug concentration in the ultrafiltrate (unbound) and C_t is the total concentration before the experiment. As for equilibrium dialysis, non-specific

binding may be high with this technique. However, the relative low cost, easiness and speed of assay have contributed to the widespread use of this technique.

10.6.3 Ultracentrifugation

With this technique, the drug–protein complex formed during incubation is determined by pelleting the complex using high speed centrifugation, leaving the free drug in the supernatant. The time and the rate of centrifugation are kept constant as far as the entire sedimentation process is completed. After centrifugation, at typically 100,000 g for some hours, an aliquot of supernatant is taken to measure the free drug concentration.

The percentage of unbound is calculated by:

$$\%\text{unbound} = \frac{C_u}{C_t} \times 100 \tag{10.13}$$

where C_u is the drug concentration in the supernatant (unbound) and C_t is the total concentration before the experiment. The applicability of this technique is limited by a series of factors: costly equipment, low sample throughput, limited volume for free assay, physical phenomena (sedimentation, back diffusion, binding to lipoproteins).

10.7 *GV150526A: a case history of a highly bound drug*

GV150526 is a potent and selective antagonist of the modulatory glycine site of the *N*-methyl-D-aspartate receptor and is in development as a possible therapy to reduce neuronal damage after cerebral ischemia stroke.

The structure of GV150526A (sodium salt) is shown in Figure 10.3. The compound is characterised by a fairly high lipophilicity (log *D* at pH 7.4 = 1.9) and a fairly acidic carboxylic acid group (pK_a 3.3) and hence it was expected to possess a reasonably high binding to albumin.

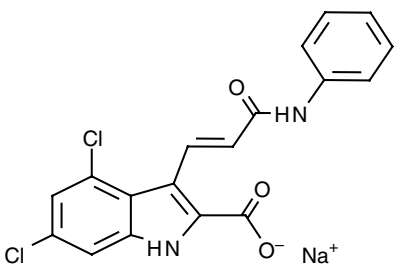

Figure 10.3 *Structure of GV150526A.*

Equilibrium dialysis was chosen as the technique of choice because of the high sensitivity required and an already known problem of high non-specific binding.

10.7.1 Non-specific binding

The standards' tests were conducted in order to establish the correct conditions for equilibrium dialysis including estimation of dialysis time to obtain equilibrium, volume shift and non-specific binding to the dialysis material. By dialysing pure solutions of GV150526 in phosphate buffer, evidence was obtained that substantial non-specific binding to the Teflon cells or dialysis membrane was present. However, this factor is not a limitation to performing equilibrium dialysis as long as the degree of non-specific binding or the amount of compound bound non-specifically, is minor compared to the amount in the plasma compartment and enough time is allowed to eventually obtain equilibrium. Plasma protein-bound compound will continually dissociate from its plasma protein binding sites until all non-specific binding sites are saturated and then the true compound bound–unbound equilibrium situation can be measured. In practice, low molecular weight endogenous dialysable material from plasma will also bind to these sites, so in practice equilibrium should be reached relatively quickly. This situation is different to that of ultrafiltration where the unbound fraction is separated from the 'reserve' of bound compound therefore any effect of non-specific binding to the apparatus or filter membrane will produce an underestimate of the free concentration.

10.7.2 Use of radiolabel

Initial attempts to determine the protein binding in plasma were made using a ^{14}C-labelled compound possessing a radiochemical purity of 98 per cent. It was immediately observed that the binding was very high, certainly greater than 99 per cent. However, when the fraction of binding approaches the radiochemical purity the effect of the radiochemical impurities cannot be ignored.

Consequently, care has to be taken when using radiolabelled compounds, without HPLC separation, to measure the binding of highly bound drugs (Honoré, 1987) as any impurity in the radiolabelled compound could have a completely different binding to the parent compound and falsify the result. Analysing the buffer after dialysis by HPLC, in fact, confirmed that the ratio of radioactive impurity (more polar than GV150526) to GV150526 after dialysis was significantly higher than in the starting compound, indicating a much lower binding of the impurity to plasma proteins. Thus the result of binding using the radiolabelled compound without HPLC separation was significantly under-estimated. Therefore all future work was performed using cold compound and HPLC methods.

10.7.3 Identification of principal binding proteins

Studies were performed to determine the main proteins involved in GV150526 binding in human plasma. Albumin, α1-glycoprotein, low and high density lipoproteins and gamma globulin were tested. Albumin was shown to be the most important protein, binding GV150526 to the extent of >99.99 per cent at clinically relevant concentrations. Binding to lipoproteins was in the range 90–94 per cent. Two binding sites to HSA were observed, one with a very high affinity (Kd_1 0.5 μM) and the second with much lower affinity (Kd_2 100 μM).

10.7.4 Linearity of protein binding

The binding of drugs to plasma proteins is usually quoted as a percentage of the total drug that is bound. This value depends on the number of binding sites available which is related to the concentration of protein and the affinity of the drug for the various proteins. The concentration of albumin, the principal binding protein on plasma has a normal concentration of around 40 mg/mL (about 550 μM). When the plasma concentration of drugs with high binding affinity to albumin increases to a level where all the available sites are occupied, then the binding is said to be saturated and the %binding will decrease with increasing plasma concentration. For GV150526 this would be expected in humans when plasma concentrations approach approximately 100 μM (affinity constant Kd_2). The saturation of binding may result in an increased clearance and/or an increased volume of distribution and may result in non-linear kinetics.

In the concentration range studied, the test compound demonstrated non-linear protein binding above certain concentrations (Figure 10.4). These concentrations were different between animals and man and also shown to be different between healthy volunteers and stroke patients. The binding in rat and dog saturated at lower concentrations compared to human volunteers. This causes a complex relationship of %unbound–total concentration between animals and man which was important to understand in the determination of toxicological cover for man (see Section 10.7.5). At lower total concentrations, there is a lower difference between man and animals than at higher concentrations ratio. For example at 75 μg/mL the ratio between rat:dog:human volunteers is 6:16:1 whereas at 150 μg/mL the ratio is 36:50:1. There is a clear difference between healthy volunteer and stroke patients, with, for patients, a lower concentration in which saturation occurs and hence a higher fraction unbound at the higher total concentration studied. This is probably due to a number of factors possibly including lower plasma albumin concentrations due to age factors (Mayersohn, 1992), co-administered medications and altered biochemical parameters due to the disease state. This difference also signifies a different relationship in protein binding between stroke patients and animals. At 75 μg/mL the ratio rat:dog:patients is 4:16:1, whereas at 150 μg/mL the ratio

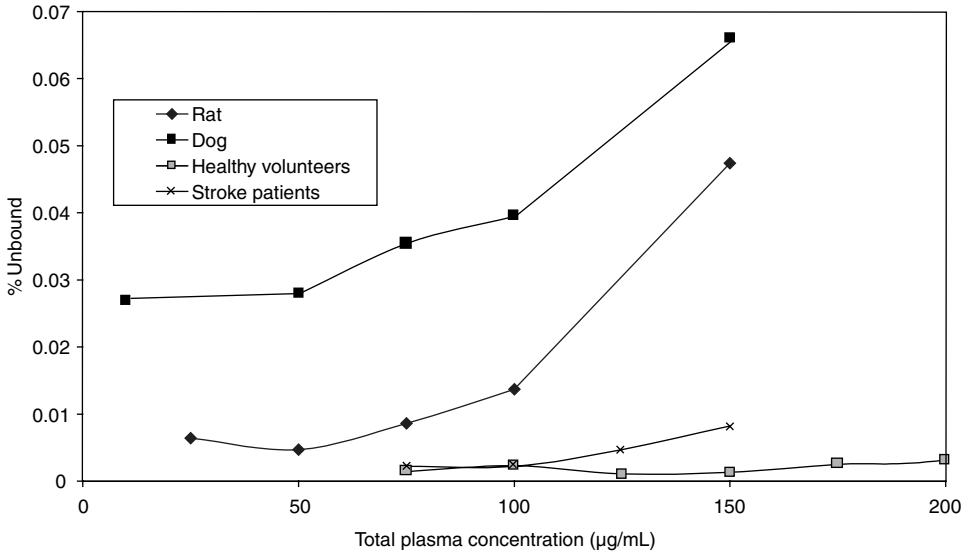

FIGURE 10.4 *Comparison of GV150526 %unbound between rat, dog, human volunteer and stroke patient's plasma.*

becomes 6:8:1, which is considerably less than the ratio between animals and healthy volunteers.

GV150526 is very similar to warfarin in its characteristics as it is a fairly lipophilic acidic compound, which is almost completely eliminated hepatically, and has a very low clearance and volume of distribution in animals and man. Therefore it should be expected that if there are differences in protein binding between animal species, then plasma clearance is also likely to differ. Where binding is very high, as in this case, small changes in %bound can produce large differences in clearance. Figure 10.5 shows the plasma protein binding of GV150526 in mouse, rat, dog, rabbit and man and their correlation with clearance. As can be seen, there is a very close correlation between the fraction unbound and clearance for GV150526, and the inter-species differences in clearance are large. Man has the lowest clearance whilst rat, dog, mouse and rabbit have 15-, 25-, 76- and 200-fold higher clearance, respectively. The inter-species differences in albumin binding may reflect differences in affinity or number of binding sites for GV150526 and are probably caused by the known differences in the amino acid sequence around the principal albumin binding sites between animal species (Lin, 1987).

10.7.5 Toxicological cover

Regarding toxicological cover, two important problems were encountered during the pre-clinical development of GV150526. First, little significant toxicity was

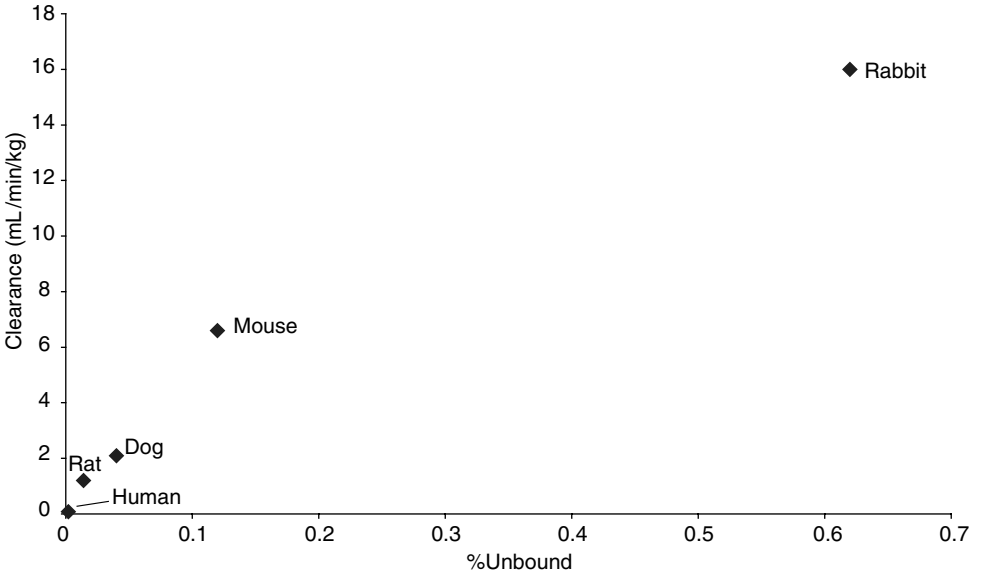

FIGURE 10.5 *Relationship between protein binding (%unbound) in plasma and clearance in toxicological species and man.*

demonstrated at the highest dose administrable in animals. This was in part due to the low intrinsic toxicity of the compound but also due to the fact that chronic testing at very high doses was limited by injection site irritancy. Second, the large differences in protein binding and clearance between the main toxicological species and man made it difficult to obtain standard toxicological cover. As the protein binding in human plasma was higher than all the species tested and consequently clearance was lower, the plasma *AUC* was always much higher in man compared to animals, at a given dose. Therefore these factors made it difficult to administer high enough doses in animals to provide enough toxicological cover for man, based on plasma total concentrations. However in this case the standard approach of using total concentrations to calculate *AUC* is not the most appropriate one. For GV150526, the target organs for toxicity were the eliminating organs, liver and kidney. It is likely that target organ toxicity initiates when a certain critical concentration is exceeded. For drugs with very low clearance and elimination half-life this critical concentration will be exceeded if dose is increased and also if plasma total clearance increases and therefore toxicity should not only be dependent on dose but also on clearance. As, in this case, clearance is highly correlated to the unbound or free fraction in plasma, drug exposure and therefore toxicity of the eliminating organs should be much more closely correlated with the free plasma concentrations rather than total concentrations. Therefore the evaluation of toxicological cover is more correctly based on free concentrations and free *AUC* for this compound. Initially, estimates of free *AUC* were obtained by measuring the free

concentration from *in vivo* samples at only the maximum concentration of the time–concentration profile and using the calculated %bound value to determine free *AUC* from the total *AUC* measured. This was done to limit the number of animals used in the study, as the determination of the protein binding of GV150526 with equilibrium dialysis requires 1 mL of plasma, due to the very high analytical sensitivity needed. However, the protein binding of this compound was shown (see Figure 10.4) to be non-linear in rats and dogs at the concentrations observed during the toxicological studies. Therefore, to more accurately estimate free *AUC* in these studies, extensive *in vitro* data on the correlation of %unbound versus total concentration were produced over the entire concentration range encountered. This data was then used to calculate (by linear interpolation) the %unbound at each concentration time point in the profile and so more accurately determine free *AUC* in toxicity studies. This approach was deemed valid as the *in vivo* and *in vitro* %unbound values were very similar, probably due to the fact that the levels of GV150526 metabolites in plasma were generally very low compared to GV150526.

As GV150526 displays restrictive plasma protein binding, it is likely that the fraction unbound will also be related to the degree of brain penetration (Robinson and Rapoport, 1986). To take this into account, the anticipated efficacious dose in man was extrapolated from the principal animal model of activity using free plasma concentrations. An efficacious dose in man was predicted when the free plasma concentration exceeded the maximum free concentration observed after a maximally efficacious dose in the rat pharmacological model. The clinical dosage regimen was then designed so that free concentrations in the clinic were always above this threshold level. Despite the very high restrictive protein binding and the consequent low brain penetration, GV150526 is very efficacious in animal models of stroke due to the high receptor affinity and low toxicity, the latter enabling the administration of very high doses.

10.7.6 Protein-binding displacement

If pharmacological activity depends on distribution into tissues, such as the CNS, a protein-binding displacement due to concomitantly administered medications could potentially cause some change in drug activity.

In the case of GV150526, where the target organs for toxicity were the eliminating organs, kidney and liver, there was much concern over the possibility of abruptly increasing free concentration and so increasing clearance. Renal damage in rats appeared to be related to free concentration. Renal toxicity was more apparent after a bolus administration, where protein binding was saturated at the high plasma concentrations obtained, compared to long infusion and strongly related to the concentration of parent GV150526 in urine. Because of these findings, potential protein-binding displacements in human plasma were studied *in vitro* in order to obtain more information about which type of drugs could

potentially alter the plasma protein binding on GV150526 in the clinic and alter toxicity. The real *in vivo* effect of protein-binding displacement by these drugs could then be tested in the clinic. Drugs studied were chosen depending on their likely concommitant administration with GV150526, their binding affinity to albumin and their therapeutic concentrations and on previous knowledge of clinically relevant protein-binding interactions. The drugs studied were warfarin, phenytoin, ibuprofen, phenylbutazone, salicylic acid, heparin and tissue plasminogen activator. Bilirubin was also included. To study a worst case situation, the median peak concentration of GV150526 observed in stroke patients was chosen. The potential displacer was tested at three different concentrations around its normal therapeutic concentration range. The results are summarised in Table 10.5. Phenytoin, ibuprofen, warfarin, heparin and tissue plasminogen activator, at their normal therapeutic concentrations, showed absolutely no displacement. Salicylic acid and phenylbutazone, on the other hand, gave a significant increase in GV150526 free concentration. A slight displacement of GV150526 binding was observed with warfarin at concentrations slightly higher than therapeutic. These results suggest that GV150526 binds to site 1 (Hervé, 1994) of albumin as phenylbutazone and warfarin bind specifically to site I whereas ibuprofen binds with high affinity to site II. The only slight effect by warfarin was probably due to the low concentration of warfarin used. GV150526 was also shown to displace warfarin at normal therapeutic concentrations and this finding caused some concern, as warfarin's action as anti-coagulant has been shown to be related to the free plasma concentration. Therefore a clinical study was performed to study the possible effects of the protein-binding displacement of GV150526 on warfarin. However, no significant effect on anti-coagulant activity was observed.

TABLE 10.5 *The study of the protein-binding displacement of GV150526 in human plasma by possible concomitant drugs, using* in vitro *equilibrium dialysis*

Displacer	Displacer concentration range (μg/mL)	Relative displacement compared to control*
Ibuprofen	20–100	0.5–0.9
Phenylbutazone (ng/mL)	50–300	1.7–12
t-PA (μg/mL)	0.1–4	0.8–1.2
Bilirubin (mM)	10–50 (mM)	0.9–1.0
Phenytoin	3–36	0.8–1.0
Salicylic acid	50–500	1.4–9.0
Warfarin	1–10	1.0–1.2
Heparin	1–100 (units)	1.0–1.1

t-PA = tissue plasminogen activator.

*Calculated from %unbound with displacing drug/%unbound without displacing drug (1.0 = no displacement).

10.8 *References*

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