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THE SPECIFICITY OF DRUG BINDING SITES ON HUMAN SERUM ALBUMIN

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Today, it is well established that the binding of drugs in serum will strongly influence the pharmacokinetic parameters of a drug, such as its distribution volume and clearance. It is also evident that the binding of the drug—in serum and elsewhere in the tissues—will have an influence on the duration and intensity of the pharmacological effect. Several excellent papers and reviews have dealt with these issues in recent years.[1-8] It is obvious that albumin, being the most abundant protein species in the extracellular fluids, is the most important drug-binding protein, although other proteins can play a pharmacokinetic role. Thus, e.g., orosomucoid (α_1-acid glycoprotein) can bind some basic and neutral drugs,[9] and lipoproteins some highly hydrophobic drugs.[10]

The primary structure of human serum albumin (HSA) is now known.[11,12] However, all efforts to study the three-dimensional structure by x-ray spectroscopy have hitherto failed, and a detailed knowledge of the mechanisms involved in the binding of drugs or endogenous compounds is still missing. The broad binding specificity of HSA is remarkable. Several compounds of widely different structure can be bound with high affinity—e.g., fatty acids, bilirubin, tryptophan, as well as many drugs. It is also striking that different reports from quantitative studies on the binding of different compounds have shown varying results, which cannot be solely explained by technical problems or different experimental conditions. All available information indicates that HSA is a highly "flexible" and "adaptable" molecule, the structure of which can be strongly influenced by different "modulating" substances. It is now well known, e.g., that free fatty acids up to a 4-fold molar excess over HSA, enhance the binding of warfarin.[13] Ca^{2+} and other small ions also can change the

conformation of HSA—and thereby, the binding properties—by affecting the N-B transition of HSA at physiologic pH values.[14,15] It is, therefore, logical to conclude that both positive and negative allosteric interactions between different binding sites can influence the binding of both endogenous and exogenous compounds. It has, moreover, been shown recently that drugs theoretically show unsaturated binding,[16] a phenomenon which earlier has been shown to be valid also for the binding of fatty acids.[17] In these situations, binding can be correctly described by a stoichiometric binding model

$$r = \frac{K_1 \times D_f + 2K_1K_2 \times D_f^2 + \cdots}{1 + K_1 \times D_f + K_1K_2 \times D_f^2 + \cdots}$$

and presented as binding isotherms.[18]

However, pharmacological doses of drugs generally are relatively small and the blood concentration does not normally reach equimolar concentration (with HSA). Essentially only one site is then active with little influence from secondary sites. There are also situations when different ligands (e.g., bilirubin and benzodiazepines) bind independently to HSA.[19] Chemical modification of HSA with N-acetylimidazole and cyclohexanedione, which preserves the protein conformation, has, moreover, shown that the binding sites for these ligands contain essential amino acids (lysine and arginine) showing the specificity of the binding interactions.[20] The existence of specific binding sites has also been shown for indomethacin, which is a drug binding to at least three different sites as studied by circular dichroism titrations.[21] In these cases, it is often justified that the well known site-binding model of Scatchard is applied,[22] in which

$$r = \frac{k_1 \times D_f}{1 + k_1 \times D_f} + \frac{k_2 \times D_f}{1 + k_2 \times D_f} + \cdots + \frac{k_i \times D_f}{1 + k_i \times D_f}$$

It should be emphasized that this equation is valid only if independent binding of ligands occurs. Correctly used, the Scatchard plot can give information on the binding constants involved.[18,23,24] The site-binding model is also a model which perceptionally is more suitable for a discussion of binding site specificity and for an understanding of drug binding displacement phenomena.[25]

Binding displacement may have pharmacokinetic significance when the binding degree is high and the distribution volume is small.[1,26] One of the first clinically relevant findings was published in 1956, when a clinical trial showed that sulfisoxazole displaced HSA-bound bilirubin which subsequently produced kernicterus in

premature infants.[27] Another clinically significant situation is the interaction between valproate and phenytoin,[28] which should be remembered when the plasma level is analyzed, so that the free fraction of phenytoin is determined.

It is thus important that the number of binding sites of HSA and the specificity of respective site are known. A convenient way to study these problems is to follow the capacity of different drugs to displace specific markers representing different binding sites. Sudlow, Birkett, and Wade[29,30] used the fluorescent probes dansylamide and dansylsarcosine (representing sites I and II, respectively). Honoré and Brodersen[25] used mono-acetyl-diamino-diphenylsulphone (MADDS) in a dialysis system. MADDS binds to the same site as dansylamide, bilirubin, and warfarin. Ekman, Lofter, and Sjöholm have used a solid phase system with HSA entrapped in microparticles of macroporous polyacrylamide and several radioactive markers, the displacement of which was measured. The binding properties of HSA are retained in the solid phase,[31] which also conveniently can be used to quantitatively determine binding constants of drug-HSA-complexes.[32] In the first study on the binding sites of HSA,[33] the displacement of diazepam, digitoxin, and warfarin, binding independently to HSA, was followed. The principle applied is shown in Fig. 4-1.

The solid phase-system is a simple and rapid system, which can be used with a low HSA-concentration—generally 0.5-2 mg HSA/

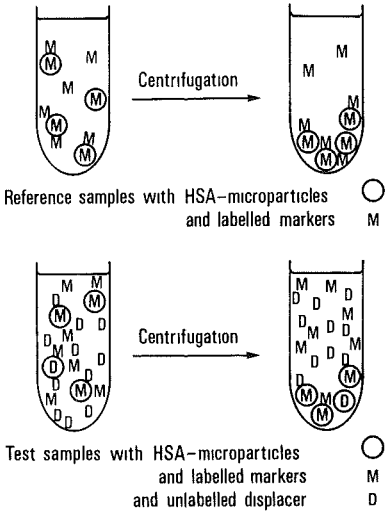


FIGURE 4-1
The principle for studying the displacement of a marker, M, from immobilized human serum albumin in microparticles by a drug, D.

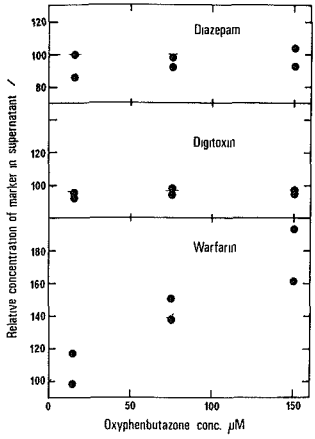


FIGURE 4-2
Displacement of diazepam, digitoxin, or warfarin (11.25 μM) from albumin in microparticles (15 μM) by oxyphenbutazone in 0.1 M NaCl and 0.005 M phosphate buffer, pH 7.4, and 25°C.

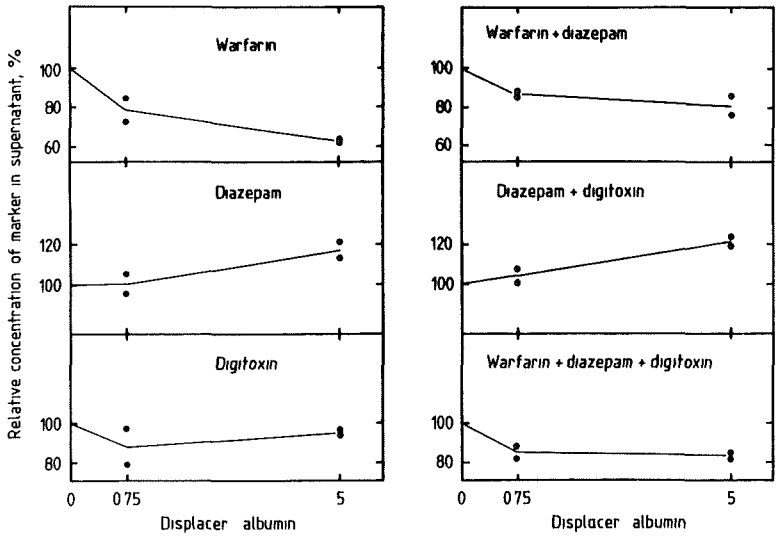
ml. It is important that the marker-HSA ratio be low (0.5-0.75), so that essentially the primary binding site for the marker is active, when binding competition is to be studied. The potential displacer is added in increasing concentration up to a displacer-HSA ratio of 5-10. The equilibrium between the ligands and the protein is attained very rapidly; the samples can be centrifuged after 5-15 min and the radioactivity is determined in the supernatant. A representative example of the results obtained is shown in Figure 4-2. The relative concentration of the respective marker in the supernatant was measured, when an increasing amount of oxyphenbutazone was added. As is evident from the figure, oxyphenbutazone effectively displaced warfarin from HSA in the solid phase, while no significant effects on the binding of the other markers were detected. The conclusion is, of course, that warfarin and oxyphenbutazone bind to a common binding site or binding region on HSA. In this way, about 150 drugs and other compounds have been studied.[33] A summary of the findings is given in Table 4.1.

For one drug, namely tamoxifen, comparison of the results obtained with the three markers led to the conclusion that there also exists at least a fourth binding site on serum albumin. Similar

TABLE 4.1
Binding of Drugs to Human Serum Albumin

Binding to:			
Diazepam Site	Digitoxin Site	Tamoxifen Site	Warfarin Site
Benzodiazepines	Acetyldigitoxin	Clomifen	Azapropazone
Cloxacillin			Azidocillin
Dicloxacillin			Chlorazepate
Dicoumarol (2)*			Chlorothiazide
Ethacrynic acid			Dicoumarol (1)*
Flucloxacillin (2)*			Diflunisal
Flurbiprofen (1)*			Flucloxacillin (1)*
Glibenclamide			Flurbiprofen (2)*
Ibuprofen (1)			Furosemide
Indomethacin			Glibenclamide
Ketoprofen			Indomethacin
Naproxen			Ketoprofen (2)*
Probenecid			Nalidixic acid
Propiomazine			Naproxen
Tamoxifen (2)*			Oxyphenbutazone
Tolazamide			Phenylbutazone
Tolbutamide			Phenytoin
Dansylsarcosine			Salicylamide
Tryptophan			Salicylazosulphapyridine
			Salicylosalicylic acid
			Sulphadimethoxine
			Sulphamethizole
			Tolbutamide
			Valproate (sodium)
			Bilirubin
			Sulfobromophthalein (2)*

*Some substances bind efficiently to more than one site. The numbers in parentheses denote primary (1) or secondary (2) sites.



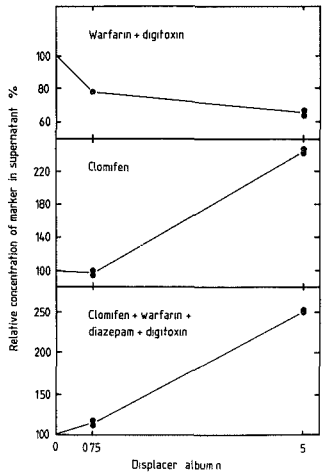


FIGURE 4-3 a, b
Displacement of tamoxifen (3.75 μM) from albumin in microparticles (7.5 μM) by different ligands as inserted, in 0.1 M KCl and 0.005 M phosphate buffer, pH 7.4, at 25°C. The ratio of displacer : albumin is related to each of the ligands used.

indications were found when the binding of sulfobromophthalein was followed. A separate study was therefore initiated, in which the binding of tamoxifen was investigated in detail.[34] It then became clear that radioactively labeled tamoxifen could not be displaced by any of the markers representing the three earlier identified binding sites. As shown in Figure 4-3, clomifen, which is structurally very similar to tamoxifen, was able to displace tamoxifen competitively either alone or in combination with other ligands. Thus, tamoxifen and clomifen reversibly react with HSA in a normal equilibrium and represent a site 4. Warfarin could not displace tamoxifen but did, in fact, increase the binding of tamoxifen and, self-evidently, tamoxifen conversely improved the binding of warfarin. Thus, it can be concluded that energetic coupling[35] also exists between the two binding sites for warfarin and tamoxifen.

It is obvious from our studies that drugs are bound to HSA in a specific way, so that groups of drugs in several instances compete for a common site or, in some instances, for two or more sites. It is also obvious that there are energetic couplings between these sites, in such a way that a ligand bound to one site can impair or improve the binding to another site binding a second ligand. This is not a unique characteristic of the albumin molecule, but is a further indication of the structural flexibility of albumin. This means in turn, that it is not necessary to postulate that preformed binding sites do exist on the protein surface. Such sites or binding areas may more probably be induced by the bound drug, the structure of which also may be changed during the binding process according to normal thermodynamic principles. Smaller or larger parts of the protein surface are then involved in the binding interaction, which may explain why, for example, chemical modification can affect the binding

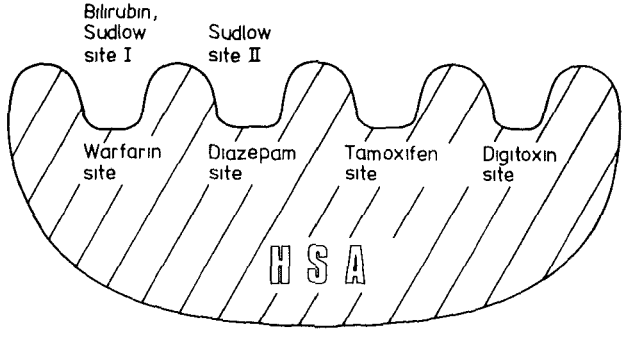


FIGURE 4-4
The binding sites of human serum albumin.

of drugs to one and the same site differently, and why drugs can affect the binding to other sites via allosteric regulations differently.

In summary, drugs can be found to at least four different sites (or regions) on HSA as schematically shown in Figure 4-4. Drugs representing the different sites do not competitively inhibit the binding to other sites, but energetic coupling mechanisms exist between the different sites. Two of the sites are responsible for the binding of a vast majority of the protein-bound drugs, while the remaining two sites—the digitoxin and tamoxifen sites—have a limited specificity.

NOTES

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