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Binding of Drugs to Human Serum Albumin:XI.[1]

The Specificity of Three Binding Sites as Studied with Albumin Immobilized in Microparticles[2]

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SUMMARY

SJÖHOLM, INGVAR, B. EKMAN, A. KOBER, I. LJUNGSTEDT-PÅHLMAN, B. SEIVING AND T. SJÖDIN: Binding of drugs to human serum albumin. XI. The specificity of three binding sites as studied with albumin immobilized in microparticles. *Mol. Pharmacol.* 16, 767-777, 1979.

Human serum albumin can be immobilized in spherical, macroporous microparticles of polyacrylamide of about 1 μm in diameter with retention of its native properties. It has been shown that diazepam, digitoxin and warfarin independently bind to albumin and can conveniently be used as markers of three separate, discrete binding sites on albumin. A simple technique has been devised by which the capacity of about 140 drugs and other compounds to affect the binding of the radioactively labeled markers has been studied. Some drugs, *e.g.* antirheumatic drugs of the isopropionic acid-type, some antidiabetic agents, penicillin derivatives, benzodiazepines, tryptophan, dansylsarcosine, and sulphobromophthalein efficiently displace diazepam. Other drugs, *e.g.*, some diuretics, sulpha drugs, phenytoin, salicylic acid and butazone derivatives, azapropazoe, bilirubin, and dansylamide displace warfarin. Displacement of digitoxin is less common. In some cases the binding of the markers is improved, *e.g.*, tamoxifen increases the binding of warfarin. Both competitive and allosteric mechanisms are responsible for the changed binding of the markers. Some results suggest the presence of more than the three binding sites for drugs on the albumin surface studied with diazepam, digitoxin and warfarin.

INTRODUCTION

The binding of drugs to proteins in the blood will strongly influence the distribution, elimination and pharmacological effect of the drug (1-4). Albumin is in most cases the predominant protein, binding drugs in blood and in the interstitial fluid (5). The binding of a drug can in several cases be modified by the presence of other drugs or endogenous substances, which can compete for the same binding site (drug "displacement" by competitive inhibition). Alternatively, two ligands can be bound to two separate sites and mutually affect the binding of each other by free energy-coupling between the two sites (6). Such allo-

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[1] For X in the series, see ref. 12.

[2] The work has been briefly presented at the 11th FEBS Meeting, Copenhagen 1977.

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steric mechanisms may be so weak that the binding can be classified as independent, as has been shown recently with diazepam and bilirubin (7), but can also result in increased (8–10) or decreased binding (11, 12). Drug interactions on the protein binding level will in most cases significantly affect the apparent distribution volume of the drugs and may also affect the rate of elimination of the drugs (3, 4). Obviously, a prerequisite for a correct prediction of whether an interaction between drugs will occur is knowledge of which sites on albumin the drugs are bound to, as has recently been observed in liver diseases (12).

HSA[3] can conveniently be immobilized in microparticles of polyacrylamide (13). The binding properties of albumin are apparently retained in the microparticles owing to the macroporous structure of the polyacrylamide gel (13). Albumin immobilized in such microparticles can therefore conveniently be used to determine the characteristics for the binding of a drug to HSA, either directly with labeled drug or indirectly via an other labeled, competing drug (14). In the present work, three labeled drugs, diazepam, digitoxin and warfarin, have been used as markers. As has been shown earlier bilirubin and diazepam bind to two separate sites independently of each other (7, 15). When this work was started it was noted that bilirubin and warfarin bind to the same primary site on HSA and digitoxin to a site separate from the other two. Thus, the markers represent three different sites. The ability of several drugs representing different pharmacological groups to displace these labeled markers has been tested.

MATERIALS AND METHODS

[^{14}C]Diazepam was synthesized from nordiazepam by methylation with [^{14}C]methyliodide (59.4 mCi/mmole) obtained from The Radiochemical Centre, Amersham, England. [^{3}H]Digitoxin (7.4 Ci/mmole), [^{14}C]salicylic acid (52 mCi/mmole), [^{14}C]tryptophan (52 mCi/mmole) and [^{14}C]warfarin (51 mCi/mmole) were also obtained from The Radiochemical Centre. [^{3}H]Digitoxin was purified by preparative thin layer chromatography prior to use. The radiochemical purity (>98%) of all the substances was checked by thin layer chromatography. Unlabeled drugs were kindly placed at our disposal by the respective manufacturer or representative in Sweden. They were used without further purification.

Human serum albumin. HSA was purchased from AB KABI, Stockholm. It contained small amounts of transferrin (<1%) as well as dimeric forms of HSA (<5%) as estimated by polyacrylamide-gel electrophoresis. No stabilizers were added and the protein was used directly for the preparation of HSA-microparticles.

Immobilization of HSA in microparticles.[4] The microparticles containing HSA were prepared by emulsion polymerization according to the modified procedure described by Ekman and Sjöholm (16). The total concentration of acrylic monomers was 8% and the fraction of bis-acrylamide was 25%. The concentration of HSA in the water phase amounted to 100 mg/ml. After the polymerization, the microparticles were washed extensively with water and buffer by repeated centrifugations.

The mean diameter of the microparticles was about 1 μm. The protein content was determined by amino acid analysis after hydrolysis in 5.9 M hydrochloric acid for 24 hr, or from the capacity of the microparticles to bind [^{14}C]salicylic acid. The latter method was based on a calibration curve obtained from equilibrium dialysis with different concentrations of HSA. Leakage of HSA from the particles was routinely checked spectrophotometrically at 279 nm. Generally, no leakage exceeding 1% of the HSA concentration could be detected in 3–4 weeks.

Procedure. The principle of the technique used to study the capacity of a drug to displace the three different markers (diazepam, digotoxin, warfarin) is summarized in Figure 1. The reference sample (500 μl) contained HSA in microparticles (1 mg/ml or 15 μM) and a labeled marker (diazepam,

[3] The abbreviations used are: HSA, human serum albumin; dansyl, 5-dimethylamino-naphthalene-1-sulphonyl.

[4] U. S. patent 4,061,466.

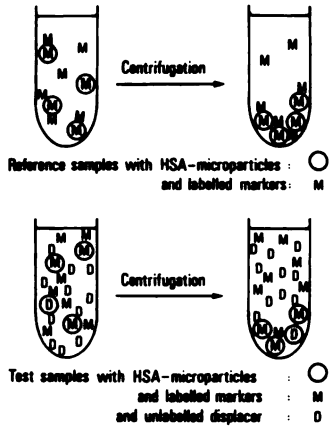

FIG. 1. *The principle for studying the displacement of a marker, M, from immobilized human serum albumin in microparticles by a drug, D*

digitoxin or warfarin, 11.25 μM corresponding to 75% of the molar concentration of HSA) in 0.005 M phosphate buffer, pH 7.4, with 0.1 M KCl. The test samples contained, in addition the displacing agent to be studied, which was added to produce drug:HSA molar ratios of 1:1, 5:1 or 10:1. Thus, the concentration of displacing drug ranged from 15 μM to 150 μM. Buffer-insoluble drugs were dissolved in a minimum amount of ethanol. All samples in a series contained the same concentration of ethanol, not exceeding 2%.

The incubations were performed in small plastic Ellerman tubes (5.5 × 1.1 cm) at room temperature (22-24°). The equilibrium for the drugs between the microparticles and the buffer is attained very rapidly and the tubes were centrifuged after about 15 min in a small table top centrifuge at about 3,000 × *g* for 20 min. Aliquots (100 μl in duplicates) were taken from the supernatants and the radioactivity determined by liquid scintillation counting in a Beckman Liquid Scintillation Counter, LS 100-C, with 5.0 ml Instagel (Packard Instrument Co). The concentration of the marker in the test samples was then related to that in the reference sample and expressed as percent. The markers do not bind to the tube nor to the polyacrylamide matrix. Glass tubes were used only when clofibrate and tryptophan were studied.

The differences between the primary and secondary association constants of the markers, as well as the concentrations of HSA and the markers chosen in this study, ascertain that any binding to secondary sites can be neglected. Thus, in the procedure described, the binding of the markers to the HSA-microparticles essentially occur to their primary binding sites.

RESULTS

Binding of labeled markers. Before any experiments with displacing drugs were performed, the binding of the labeled markers to the HSA-microparticles was analyzed. The results, analyzed according to Scatchard (17), gave binding constants coinciding with those earlier obtained with diazepam (18×10^5 M^{-1}) and warfarin (21×10^5 M^{-1}) with microparticles (14) or by equilibrium dialysis (14) and by circular dichroism titration (18). The binding of digitoxin closely corresponded to a binding constant of 9×10^6 M^{-1} earlier reported by Lukas and Martino (19) and by us (20) from equilibrium dialysis studies. The binding characteristics obtained with the immobilized HSA are thus similar to those seen with soluble HSA.

The binding of the markers varied slightly with the ethanol concentration. Under the conditions used, the binding constants signify that the binding degree amounted to about 55, 37 and 65% for diazepam, digitoxin and warfarin, respectively, in the reference samples containing 2% ethanol.

In initial tests, it was confirmed that bilirubin and warfarin displaced each other from HSA, also at ligand-HSA ratios <1. In addition, it was confirmed that the binding of neither diazepam nor warfarin was affected by the presence of the other at lower concentrations. However, when the ligand-HSA ratio exceeded one, a free energy-coupling between the diazepam and warfarin sites was observed, resulting in increased binding, confirming earlier findings (7).

It was also noted in the initial tests that neither diazepam nor warfarin displaced digitoxin from its binding site on HSA under the conditions described under PROCEDURE above. It thus became evident that

digitoxin may demonstrate a third binding site on HSA in addition to the two occupied by diazepam and warfarin.

Displacement of markers by drugs. The ability of about 140 different drugs to displace the labeled markers from the HSA-microparticles has been tested. The drugs belong to several different pharmacological groups with varying chemical structures. Figures 2–5 show representative examples of the types of results obtained.

Figure 2 exemplifies a simple displacement mechanism. Oxyphenbutazone displaces warfarin from its binding site without affecting the binding of the other markers. Similarly, sulphadimethoxine is a potent inhibitor of the binding of warfarin (Fig. 3), but concomitantly the binding of the other markers is slightly decreased. The pronounced increase of free warfarin in the supernatant indicates that both oxyphenbutazone and sulphadimethoxine competitively displace warfarin. However, the results do not allow definite conclusions to be drawn about the mechanisms responsible for the small release of diazepam and digitoxin seen with higher concentrations of sulphadimethoxine. The displacement can be due to allosteric mechanisms or to competition from secondarily bound ligand.

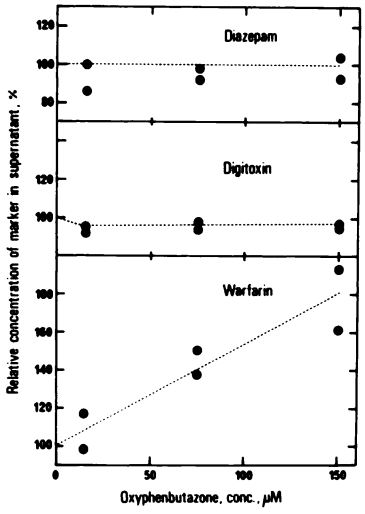

FIG. 2. *Displacement of diazepam, digitoxin or warfarin (11.25 μM) from albumin in microparticles (15 μM) by oxyphenbutazone in 0.1 M NaCl and 0.005 M phosphate buffer, pH 7.4, and 25°*

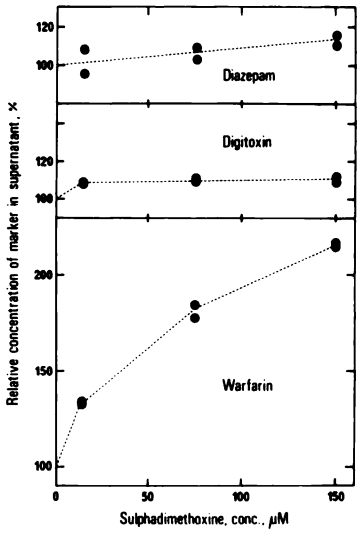

FIG. 3. *Displacement of diazepam, digitoxin or warfarin (11.25 μM) from albumin in microparticles (15 μM) by sulphadimetoxine in 0.1 M NaCl and 0.005 M phosphate buffer, pH 7.4, and 25°*

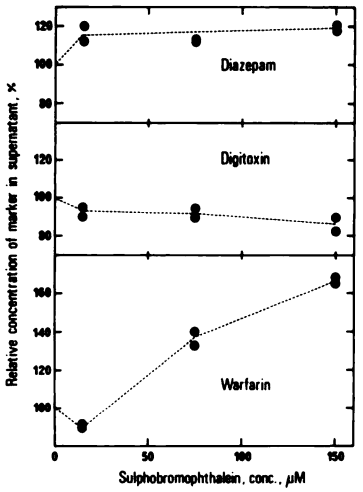

FIG. 4. *Displacement of diazepam, digitoxin or warfarin (11.25 μM) from albumin in microparticles (15 μM) by sulphobromophthalein in 0.1 M NaCl and 0.005 M phosphate buffer, pH 7.4, and 25°*

Figures 4 and 5 give more conclusive evidence that allosteric mechanisms are operating, *i.e.*, that energetic couplings exist between different binding sites. Evidently, sulphobromophthalein (Fig. 4), known to be bound to several sites on HSA (21), primarily interacts with a site on HSA in

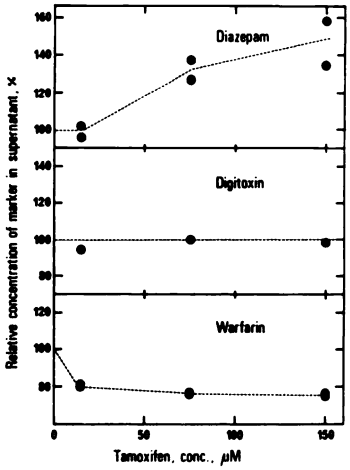

FIG. 5. *Displacement of diazepam, digitoxin or warfarin (11.25 μM) from albumin in microparticles (15 μM) by tamoxifen in 0.1 M NaCl and 0.005 M phosphate buffer, pH 7.4, and 25°*

such a way that the binding of diazepam is impaired while that of warfarin is improved. After the first site is saturated, sulphobromophthalein then can interact with the warfarin site, probably competitively. Moreover, the digitoxin binding was successively increasing with increasing concentrations of sulphobromophthalein. The results suggest that other binding sites are available on HSA than those represented by the three markers used. Further indications for such a possibility can be found in Figure 5, which shows the results obtained with tamoxifen.

The first molecule of tamoxifen apparently does not bind to any of the sites on HSA represented by the markers, as no decreased binding of the markers can be detected up to an equimolar concentration of tamoxifen. On the contrary, the binding of warfarin is highly improved (relatively by 20%), owing to a positive energetic coupling between the primary tamoxifen site and the warfarin site. However, the next molecule of tamoxifen probably binds to the diazepam site, as diazepam was strongly displaced by increasing tamoxifen concentrations.

Binding of other markers. In some drug interaction studies tryptophan (22) or dansylamide and dansylsarcosine (23, 24) have been used as markers, and their binding to HSA in microparticles was therefore studied in some detail. Figure 6 shows the results obtained when the displacement of [^{14}C]tryptophan by warfarin, diazepam and salicylic acid was studied. The binding of tryptophan under standard conditions was, however, too low to give precise results, and the concentration of HSA had to be increased 4 times to 60 μM, when about 25% of the [^{14}C]tryptophan was initially bound. As seen from Figure 6, both diazepam and salicylic acid efficiently compete with tryptophan for its primary site already at low concentrations, while warfarin displaces tryptophan only at higher concentrations.

Figure 7 shows that dansylsarcosine primarily affects the binding of diazepam and secondarily that of warfarin. Dansylamide (Fig. 8) apparently is bound to both the warfarin and the diazepam site. The binding of dansylamide to HSA is obviously comparatively weak, but as the displacement of warfarin is more effective and this drug has a higher association constant than diazepam, the warfarin site is probably the primary binding site for dansylamide.

All the results obtained are summarized in Table 1, where the drugs are arranged according to their main pharmacological effect.

DISCUSSION

The increased knowledge of the binding of drugs and endogenous compounds to

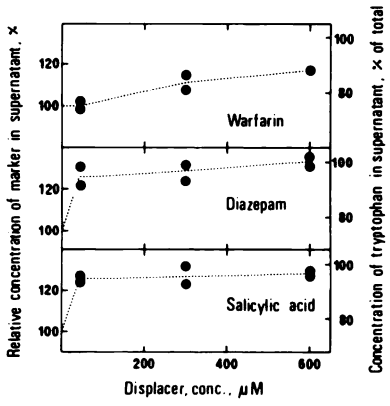

FIG. 6. *Displacement of [^{14}C]tryptophan (45 μM) from albumin in microparticles (60 μM) by diazepam, salicylic acid and warfarin in 0.1 M NaCl and 0.005 M phosphate buffer, pH 7.4, and 25°*

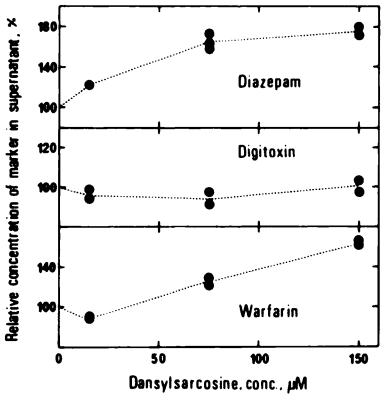

FIG. 7. *Displacement of diazepam, digitoxin or warfarin (11.25 μM) from albumin in microparticles (15 μM) by dansylsarcosine in 0.1 M NaCl and 0.005 M phosphate buffer, pH 7.4, and 25°*

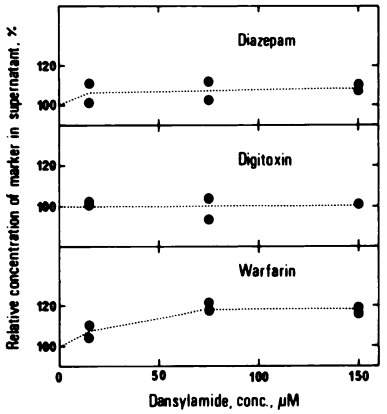

FIG. 8. *Displacement of diazepam, digitoxin or warfarin (11.25 μM) from albumin in microparticles (15 μM) by dansylamide in 0.1 M NaCl and 0.005 M phosphate buffer, pH 7.4, and 25°*

HSA and the increasing evidence for the presence of active binding sites on the HSA molecule for ligands (25) have focused the interest on the specificity of these sites. Such knowledge is obviously necessary to prove or foresee drug interactions on the protein binding level and to predict any effects of a disease state on the protein binding of a drug (11, 12, 20). These aspects have been the subject of some recent reports. Thus, Brodersen (26) has developed a sensitive technique for the determination of free bilirubin in the presence of albumin. Several drugs—*e.g.*, sulpha drugs and salicylic acid derivatives—have been shown to interact competitively with the binding of bilirubin (26). Brodersen has also used radioactively labeled diazepam and monoacetyl-diamino-diphenylsulphone (representing the primary binding site of bilirubin) in a dialysis technique to measure the competitive interaction with different drugs (27). Sudlow, Birkett and Wade (23, 24) have used dansylamide and dansylsarcosine as fluorescent probes to characterize two binding sites on HSA. The capacity of several drugs to displace the probes from their binding site was measured. Also, L-tryptophan has been used for the same purpose (22).

The technique used in the present work with HSA immobilized in microparticles has obvious practical advantages, provided the immobilization does not change the native properties of HSA. In fact, no changes of the biological properties of HSA or other proteins in microparticles have been detected (13, 14, 16). The technique is rapid and can be used with high precision, when labeled markers are available. The main sources of error are bad sampling of the HSA-microparticles (leading to varying HSA-concentrations), imperfect centrifugation (leading to too high radioactivity in the supernatant) or precipitation of the displacers or markers in the samples. When the microparticles are used carefully, the experimental error is however small. In four different series of experiments, when the binding of diazepam and warfarin to HSA-microparticles was determined, the coefficient of variation ($s_{\%}$, n = 12) ranged between 1.47 and 2.73%.

The results obtained should essentially be regarded as qualitative: limited concentration ranges were used with the displacers and the effects have not been studied systematically at the therapeutic serum concentrations. However, some conclusions relating to quantitative effects can be drawn, if one assumes that competitive mechanisms are responsible for the displacement of a marker. Thus, it is easy to define the conditions necessary to cause a 10% increase of the free fraction of the marker. This will depend on the concentrations and the relative association constants of the displacer and the markers. Taking diaze-

pam (K_a = 18 × 10^5 M^{-1}) as an example, 10% displacement will be affected by all ligands having a K_a = 1.7 × 10^4 M^{-1} at a concentration of 15 μM (*i.e.*, a ligand-HSA ratio of 1:1). The affinity of a ligand for the warfarin site has to be higher, and for the digitoxin site, lower, to achieve the same displacement, as the association constants of warfarin and digitoxin are larger and smaller, respectively.

The technique with immobilized HSA can conveniently be used for an extensive screening of potential inhibitors of the binding of a particular drug. At the same time a survey of the specificity of the albumin binding sites can be obtained showing the types of compounds that can be bound to the respective site. Information on the mechanisms causing the displacement or the increased binding can also sometimes be obtained, particularly when the results with all the markers are compared. In Table 2, a summary of the results are given, showing the site many agents or groups of agents are bound to. Only drugs displacing more than 10% of the markers at a drug-protein ratio of 1:1 are included.

As discussed above, the drugs summarized in Table 2 have association constants of about 1.7 × 10^5 M^{-1} or larger, which means that the protein binding will have significant influence on the distribution of the drugs *in vivo* (1, 2). Competition between two or more drugs for the same binding site can also be of pharmacokinetic significance, *e.g.*, affect the distribution, the elimination and thereby the time course of the pharmacological effects. However, the clinical significance of such interactions will also depend on other important pharmacokinetic factors as, for instance, the respective blood levels of the drugs, the apparent distribution volumes and the elimination mechanisms. These have to be evaluated for each pair of drugs. Table 2 thus contains lists of drugs that can interact *in vivo*. Drugs not included in the table will probably not show clinically significant interactions.

It is obvious from Table 2 that site 1 and site 3 are the more important drug binding sites, site 1 being more specific than 3. To site 1, for which diazepam was used as a marker, the benzodiazepines, some antidiabetics and antibiotics are bound together with tryptophan and strongly bound analgesic agents containing isopropionyl side chains like flurbiprofen and naproxen. Site 2 is quite specific for digitoxin and acetyldigitoxin and only small effects on this site, which probably were of allosteric nature, were detected with the drugs studied in this work. Site 3, which is the primary binding site for warfarin and bilirubin, has a broad specificity. Several strongly protein bound drugs, *e.g.*, sulpha and penicillin derivatives and analgesic agents like oxyphenbutazone and diflunisal, bind primarily to this site.

The results summarized in Table 2 coincide well with the results presented by Brodersen (26) on the binding of drugs to the bilirubin high-affinity site of HSA. It should be stressed that the table includes only drugs displacing more than 10% of the markers at a 1:1 molar ratio with respect to the HSA concentration. This means that the drugs should have K_a > about 2 × 10^4 M^{-1} when bound to the diazepam or warfarin sites, as discussed above.

Dansylsarcosine and dansylamide used by Sudlow *et al.* (23, 24) as fluorescent markers bind to the diazepam and warfarin site, respectively. Only dansylsarcosine, however, binds strongly enough to cause a significant displacement of our markers, which have higher affinity to their respective primary sites than the dansyl derivatives. Consequently, diazepam and warfarin will give more clear-cut and discriminating results when drugs with high and pharmacokinetically significant association constants are studied.

Several of the drugs listed in Table 2 are used at therapeutic blood concentrations, which are high enough to significantly displace each other from their binding sites, when given combined to a patient. The consequences will be larger apparent distribution volumes, increased total blood clearance and–depending on the principal mechanisms for the elimination of the drugs–a changed biological half-life, $t_{1/2}$. To obtain the wanted therapeutic effects, the monitoring of those drugs involved in expected interaction processes should therefore be based on the concentration of the free drug.

TABLE 1

Displacement of [^{14}C]diazepam, [^{3}H]digitoxin and [^{14}C]warfarin from human serum albumin in microparticles by drugs

HSA-microparticles (15 μM) were suspended in 0.1 M NaCl and 0.005 M phosphate buffer, pH 7.4, at 25° with the radioactive marker (11.25 μM). The drugs were added in increasing amounts and the radioactivity in the supernatant was measured and related to that obtained in the absence of the displacer. + and ++ mean >10% displacement of the marker at drug-HSA ratios of 10:1 and 1:1, respectively. - and -- mean increased binding (>10%) at drug-HSA ratios of 10:1 and 1:1, respectively. 0 means no effect (<10%). When two symbols are given, the first one means the result at 1:1 and the second one at 10:1 molar ratios.

Drug	Marker			Drug	Marker		
	Diazepam	Digitoxin	Warfarin		Diazepam	Digitoxin	Warfarin
Drugs Acting on the Cardiovascular System				*Drugs with Antihistamine Activity*			
Acetyldigitoxin	0	++	0	Chlorcyclizine	0	0	+
Digitoxin	0		0	Chlorphenoxamine	0	0	0
Procainamide	0	0	0	Cyproheptadine	0	0	0
Quinidine	0	0	0	Diphenhydramine	0	0	0
Verapamil	0	0	0	Promethazine	0	+	0
				Chemotherapeutics			
Alprenolol	0	0	0	Salicylazosulphapyridine	0	0	++
Atenolol	0	0	0	Sulphadimethoxine	+	+	++
Metoprolol	0	0	0	Sulphafurazole	0	+	++
Pindolol	0	0	--	Sulphamethizole	0	+	++
Practolol	0	0	0	Sulphamoxole	0	+	++
Propranolol	0	0	0				
				Azidocillin	+	-	++
Azapetine	0	0	0	Benzylpenicillin	0	0	0
Bethanidine	0	0	0	Cloxacillin	++	0	+
Clofibrate (ethyl ester)	+	+	0	Dicloxacillin	++	0	+
Dipyridamole	0	0	0	Flucloxacillin	++	-	++
Etilefrine	0	0	0	Clindamycin	0	+	0
Etofylline	+	0	0	Erythromycin	0	0	0
Mecamylamine	0	0	0	Fusidic acid	+	+	0
Methyldopa	0	0	0	Oxytetracycline	0	0	0
Nicotinyl alcohol	0	0	0				
Pentifylline	0	0	0	Aminosalicylic acid	0	0	0
Prenylamine	+	0	-	Cycloserine	0	0	0
Terodiline	0	0	0	Isoniazid	0	0	0
Tolazoline	0	0	0	Nalidixic acid	+	0	++
Trimetaphan camphorsulphonate	0	+	+	Trimethoprim	0	0	0
Xanthinol nicotinate	0	0	+	*Drugs Acting on the Endocrine System*			
				Propylthiouracil	0	0	+
Diuretics							
Amiloride	0	0	0	Acethohexamide	+	+	+
Bendroflumethiazide	0	0	0	Carbutamide	0	0	+
Bumetanide	0	0	++	Chlorpropamide	+	+	++
Chlorothiazide	0	0	++	Glibenclamide	++	0	++
Chlorthalidone	0	0	0	Glibornuride	+	0	+
Clopamide	0	0	0	Glymidine	0	0	+
Cyclopenthiazide	0	0	0	Metformin	0	0	0
Ethacrynic acid	++	0, -	0, +	Phenformin	0	0	0
Furosemide	0	0	++	Tolazamide	++	0	++
Methyclothiazide	0	+	0	Tolbutamide	++	0	++

TABLE 1—*Continued*

Drug	Marker		
	Diazepam	Digitoxin	Warfarin
Hypnotics, Sedatives and Tranquilizers			
Phenobarbital	0	0	0
Chlorazepate	++	0	0
Chlordiazepoxide	++	0	0
Diazepam		0	-
Nitrazepam	+	0	0
Oxazepam	++	0	0
Alimemazine	+	+	-
Chlorpromazine	+	0	-
Chlorprothixene	+	+	+
Clopenthixol	+	+	-
Flupenthixol	+	+	0
Methaqualone	0	0	0
Periciazine	+	+	0
Propiomazine	++	+	-
Prothipendyl	0	0	0
Anticonvulsants, Antiemetics and Centrally Acting Muscle Relaxants			
Carbamazepine	0	0	0
Chlorzoxazone	+	0	+
Clonazepam	0	0	-
Ethosuximide	0	0	0
Ethotoin	0	0	+
Meclozine	0	0	0
Mephenytoin	0	0	0
Paramethadione	0	+	0
Phensuximide	0	0	0
Phenytoin	0	+	++
Primidone	0	0	0
Sodium valproate	+	0	++
Trimethadione	0	+	0
Antidepressive Agents			
Amitriptyline	+	0	0
Clomipramine	+	0	0
Imipramine	0	0	0
Nortriptyline	0	0	0
Opipramol	+	0	-
Protriptyline	+	0	-
Non-narcotic Analgesics			
Acetylsalicyclic acid	+	0	+
Diflunisal	+	+	++
Salicylic acid	+	0	+
Salicylamide	+	0	++
Salicylosalicylic acid	+	0	++
Oxyphenbutazone	0	0	++
Phenylbutazone	0, +	0	++
Propyphenazone	0	+	0
Paracetamol	0	+	0
Phenacetin	0	0	0
Azapropazone	0	0	++
Flurbiprofen	++	+	0, ++
Ibuprofen	++	+	0, +
Ketoprofen	++	+	0, ++
Naproxen	++	0	++
Indomethacin	++	0	++
Miscellaneous			
Aldesulphone	0	0	0
Dapsone	0	0	0
Dicoumarol	0, ++	0	++
Warfarin	-	0	
Ethylmorphine	0	0	0
Pholcodine	0	0	0
Bromhexine	0	0	0
Chloroquine	0	0	0
Dichlorphenamide	0	-, +	+
Metrizoic acid	0	0	0
Pipazethate	+	+	++
Probenecid	++	0	+
Proxiphylline	0	0	0
Sodium cromoglycate	0	0	+
Tamoxifen	0, ++	0	- -
Dansylamide	(+)	0	+
Dansylsarcosine (piperidine salt)	++	0	+
Sulphobromophthalein	+	-	-, ++
Tris(2-butoxyethyl)-phosphate	+	0	0
Tryptophan[a]	++	0	+

[a] In this test the concentrations of HSA, ligand and markers were 4 times higher than normal.

TABLE 2

Binding of Drugs to Human Serum Albumin

Binding to:		
Site 1 (diazepam site)	Site 2 (digitoxin site)	Site 3 (warfarin site)
Benzodiazepines	Acetyldigitoxin	Azapropazone
Cloxacillin		Azidocillin
Dicloxacillin		Bumetanide
Dicoumarol (2)[b]		Chlorazepate
Ethacrynic acid		Chlorothiazide
Flucloxacillin (2)[b]		Chlorpropamide
Flurbiprofen (1)[b]		Dicoumarol (1)[b]
Glibenclamide		Diflunisal
Ibuprofen (1)[b]		Flucloxacillin (1)[b]
Indomethacin		Flurbiprofen (2)[b]
Ketoprofen		Furosemide
Naproxen		Glibenclamide
Probenecid		Indomethacin
Propiomazine		Ketoprofen (2)[b]
Tamoxifen (2)[b]		Nalidixic acid
Tolazamide		Naproxen
Tolbutamide		Oxyphenbutazone
Dansylsarcosine		Phenylbutazone
Tryptophan		Phenytoin
		Pindolol[a]
		Pipazethate
		Salicylamide
		Salicylazosulphapyridine
		Salicylosalicylic acid
		Sulphadimethoxine
		Sulphafurazole
		Sulphamethizole
		Sulphamoxole
		Tamoxifen[a, b]
		Tolbutamide
		Valproate (sodium)
		Bilirubin
		Sulphobromophthalein (2)[b]

[a] The drugs caused 10% *increase* of the binding.

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

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REFERENCES

1. Martin, B. K. Potential effect of the plasma proteins on drug distribution. *Nature,* **207:** 274–276, 1965.
2. Jusko, W. J. and M. Gretch. Plasma and tissue protein binding of drugs in pharmacokinetics. *Drug Metab. Rev.* **5:** 43–140, 1976.
3. Levy, G. and A. Yacobi. Effect of plasma protein binding on elimination of warfarin. *J. Pharm. Sci.* **63:** 805–806, 1974.
4. Wilkinson, G. R. and D. G. Shand. A physiological approach to hepatic drug clearance. *Clin. Pharm. Ther.* **18:** 377–390, 1975.
5. Peters, T., Jr. Serum albumin, in *The Plasma Proteins,* edited by F. W. Putnam. Academic Press, New York, ed 2, 133–181, 1976.
6. Weber, G. Energetics of ligand binding to proteins. *Adv. Protein Chem.* **29:**1–83, 1975.
7. Brodersen, R., T. Sjödin, and I. Sjöholm. Independent binding of ligands to human serum albumin. *J. Biol. Chem.* **252:** 5067–5072, 1977.
8. Chakrabarti, S. K., R. Laliberté, and J. Brodeur. Influence of long-chain free fatty acids on the binding of warfarin to bovine serum albumin. *Biochem. Pharmacol.* **25:** 2515–2521, 1976.
9. Nielsen, O. G., L. Storstein, and S. Jacobsen. Effect of heparin and fatty acids on the binding of quinidine and warfarin in plasma. *Biochem. Pharmacol.* **26:** 229–235, 1977.
10. Wilding, G., R. C. Feldhoff, and E. S. Vesell. Concentration-dependent effects of fatty acids on warfarin binding to albumin. *Biochem. Pharmacol.* **26:** 1143–1146, 1977.
11. Sjöholm, I., A. Kober, I. Odar-Cederlöf, and O. Borgå. Protein binding of drugs in uremic and normal serum: The role of endogenous binding inhibitors. *Biochem. Pharmacol.* **25:** 1205–1213, 1976.
12. Kober, A., Å. Jenner, I. Sjöholm, O. Borgå, and I. Odar-Cederlöf. Differentiated effects of liver cirrhosis on the albumin binding sites for diazepam, salicylic acid and warfarin. *Biochem. Pharmacol.* **27:** 2729–2735, 1978.
13. Ekman, B., C. Lofter, and I. Sjöholm. Incorporation of macromolecules in microparticles: preparation and characteristics. *Biochemistry* **15:** 5115–5120, 1976.
14. Kober, A., B. Ekman, and I. Sjöholm. Direct and indirect determination of binding constants of drug-protein complexes with microparticles. *J. Pharm. Sci.* **67:** 107–109, 1978.
15. Roosdorp, N., B. Wänn, and I. Sjöholm. Correlation between arginyl residue modification and benzodiazepine binding to human serum albumin. *J. Biol. Chem.* **252:** 3876–3880, 1977.
16. Ekman, B. and I. Sjöholm. Improved stability of proteins immobilized in microparticles prepared

by a modified emulsion polymerization technique. *J. Pharm. Sci.* **67**: 693–696, 1978.
17. Scatchard, G. The attraction of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.* **51**: 660–692, 1949.
18. Sjödin, T., N. Roosdorp, and I. Sjöholm. Studies on the binding of benzodiazepines to human serum albumin by circular dichroism measurements. *Biochem. Pharmacol.* **25**: 2131–2140, 1976.
19. Lukas, D. S. and A. G. De Martino. Binding of digitoxin and some related cardenolides to human plasma proteins. *J. Clin. Invest.* **48**: 1041–1053, 1969.
20. Kober, A., I. Sjöholm, O. Borgå, and I. Odar-Cederlöf. Protein binding of diazepam and digitoxin in uremic and normal serum. *Biochem. Pharmacol.* **28**: 1037–1042, 1979.
21. Deutschmann, G., M. Gratzl, and V. Ullrich. Binding of bromosulphthalein to serum albumin. *Biochim. Biophys. Acta* **371**: 470–481, 1974.
22. Müller, W. E. and U. Wollert. Influence of various drugs on the binding of L-tryptophan to human serum albumin. *Res. Commun. Chem. Pathol. Pharmacol.* **10**: 565–568, 1975.
23. Sudlow, G., D. J. Birkett, and D. N. Wade. The characterization of two specific drug binding sites on human serum albumin. *Mol. Pharmacol.* **11**: 824–832, 1975.
24. Sudlow, G., D. J. Birkett, and D. N. Wade. Further characterization of specific drug binding sites on human serum albumin. *Mol. Pharmacol.* **12**: 1052–1061, 1976.
25. Sjöholm, I. Binding of drugs to human serum albumin. *Proc. XIth FEBS Meeting*, Copenhagen 1977, **50**: 71–78, 1978.
26. Brodersen, R. Competitive binding of bilirubin and drugs to human serum albumin studied by enzymatic oxidation. *J. Clin. Invest.* **54**: 1353–1364, 1974.
27. Brodersen, R. Binding of bilirubin and other ligands to human serum albumin. Proc. XIth FEBS Meeting, Copenhagen 1977. **50**: 61–70, 1978.