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Classification of Drugs on the Basis of Bilirubin-Displacing Effect on Human Serum Albumin

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The classification of drugs on the basis of binding sites on human serum albumin was studied by means of displacement experiments with bilirubin. The displacement of bilirubin by various drugs was evaluated through the kinetic measurement of free bilirubin concentration based on oxidation with hydrogen peroxide and peroxidase. The drugs tested could be classified into three groups, I, II and III. Oxyphenbutazone, phenylbutazone, sulfinpyrazone, ketophenylbutazone, glibenclamide, tolbutamide, sulfisoxazole, warfarin, salicylic acid and furosemide (group I drugs) efficiently displaced bilirubin. Flufenamic acid, mefenamic acid, ibuprofen, acetohexamide and ethacrynic acid (group II drugs) did not displace bilirubin at a low molar ratio (drug/albumin < 1.5), but did displace it at a high molar ratio. Clofibrate, buformin and phenytoin (group III drugs) did not displace bilirubin. These results suggested that (1) the primary binding site and secondary binding site for the group I drugs were identical or very close to the bilirubin-binding site, (2) the primary binding site of the group II drugs was independent of the bilirubin-binding site, but a secondary site was close to it, (3) the binding site of the group III drugs was independent of the bilirubin site.

Keywords—protein binding; binding site; human serum albumin; bilirubin; warfarin; horseradish peroxidase

Albumin is the most abundant protein in blood plasma, and is able to bind, and thereby transport, various compounds such as fatty acids, bilirubin, tryptophan, steroids and a large number of drugs. In the case of drugs, the binding to albumin is a critical factor affecting the intensity and duration of drug action.¹⁻³⁾ The unbound drug in plasma is considered to be responsible for drug action. In other words, the correct therapeutic plasma level is not the total drug concentration in plasma but the concentration of the unbound portion of the drug. The binding of a drug can be modified by the presence of other drugs or endogenous substances which can compete for the same or an adjacent binding site. These phenomena strongly influence the distribution, elimination and pharmacological effects of drugs.⁴⁾

Recently, the primary binding sites for drugs have been divided into several groups by using various spectroscopic methods, specific fragmentation methods and chemical modifications of specific amino acid residues of albumin.⁵⁻¹⁰⁾ Sudlow *et al.*⁵⁾ have suggested two specific binding sites on human serum albumin (HSA), Site I and Site II, for anionic drugs on the basis of the fluorescence technique using specific probes. Ozeki *et al.*⁸⁾ have also classified the binding sites into R-site and U-site on the basis of the reaction rate of *p*-nitrophenyl acetate with HSA. Brodersen *et al.*¹⁰⁾ have suggested that bilirubin and benzodiazepine derivatives (1 mol) bind to different sites on defatted HSA. However, present knowledge does not allow the complete assignment of different drugs to high-affinity binding sites of albumin.

In the previous study,¹¹⁾ we showed that the classification of drugs on the basis of binding site was possible through competitive displacement experiments for bilirubin with various drugs. Bilirubin is a suitable binding site marker, because it has one high affinity site, whose

location within albumin is on the part of loop 3—4 including Lys-240^{12,13)} in the model of the secondary structure of HSA,¹⁴⁾ and free bilirubin concentration can be determined by the use of peroxidase and hydrogen peroxide, simply by measuring the absorbance change. However, the previous study¹¹⁾ could not characterize the high affinity binding site, since the experiments were carried out at a high molar ratio of drug to albumin. In the present study, by using the displacement of bilirubin with drugs at a low ratio of drug to albumin, we investigated in further detail the classification of primary and secondary binding sites of drugs on albumin.

Experimental

Materials—HSA (Fraction V, essentially fatty acid-free), horseradish peroxidase (Type I), bilirubin, phenylbutazone and warfarin were purchased from Sigma Chem. Co. Salicylic acid and phenytoin were obtained from Nakarai Chem. Co. Sulfoxazole and tolbutamide were obtained from Yamanouchi Seiyaku and Ono Yakuhin, respectively. The following drugs were kindly provided by the manufacturers and were of JP grade: oxyphenbutazone from Nihon Sieber Hegner; sulfipyrazone from Nihon Ciba-Geigy; ketophenylbutazone from Kyowa Hakko Kogyo; glibenclamide and buformine from Yamanouchi Seiyaku; mefenamic acid from Sankyo; ibuprofen and clofibrate from Kaken Kagaku; acetohexamide from Shionogi; furosemide from Shizuoka Caffein; ethacrynic acid from Nihon Merck Banyu. All other reagents were commercial products of special grade.

Method—The experimental method was that of Jacobsen¹⁵⁾ and Brodersen.¹⁶⁾ Briefly, HSA (108.05 mg) was dissolved in 25 ml of distilled water and the pH was adjusted to about 9.0 with 0.5 N NaOH. Bilirubin (4.0 mg) was dissolved in 200 μ l of 0.5 N NaOH and 0.5 ml of water. A portion of this solution was immediately mixed with the albumin solution and the pH was adjusted to 7.4 with 1.0 N HCl. The molar ratio of bilirubin to albumin was 0.6. The mixture was kept in the dark and stored in a refrigerator.

Drugs soluble in water were dissolved in 133 mM phosphate buffer (pH 7.4) containing the same molar amount of NaOH. One ml of albumin-bilirubin solution and 1.0 ml of the drug solution were placed in a 1.0 cm cell in a spectrophotometer with a thermostatic cell holder set at 37°C. For this, a Hitachi 557 type spectrophotometer provided with a Haake circulator (F2 Model) was used. Drugs insoluble in water were dissolved in ethanol or dimethyl sulfoxide. One ml of albumin-bilirubin solution and 1.0 ml of 133 mM phosphate buffer solution were placed in a 1.0 cm cell, and then as little as 5.0 μ l of drug solution prepared in an organic solvent was added to minimize the effects of organic solvent on the binding of bilirubin and drug to albumin, and on the enzyme reaction.

Then 5.0 μ l of 44 mM hydrogen peroxide was added to this mixture and the absorbance was recorded at 455 nm, which is close to the spectral maximum of albumin-bilirubin complex. After 3 min, an aliquot of 25 μ M peroxidase was added and the progress of the reaction was immediately monitored by recording the absorbance change.

Kinetic Procedure—Bilirubin is bound almost exclusively to one high-affinity site on the albumin molecule with a binding constant of about 10^8 M^{-1} ,¹⁷⁾ and the Michaelis constant is high compared with the free bilirubin concentration in the system. The velocity, V , of oxidation was determined with and without drug. The ratio of these velocities was equated with the ratio of free bilirubin concentration, b . The time, $t_{0.2}$, required for completion of the fraction 0.2 of the total process was measured in each experiment.

The ratio of time periods with and without drug is consequently equal to the inverse ratio of the initial velocities.

$$\frac{b}{b_0} = \frac{V}{V_0} = \frac{t_{0.2(0)}}{t_{0.2}} \quad (1)$$

Subscript 0 denotes the absence of drug. The peroxidase concentration was adjusted to give $t_{0.2(0)}$ values of about 3 min. In the presence of drug, the enzyme concentration was lowered to obtain $t'_{0.2}$ of about 3 min, since the value of $t_{0.2}$ decreases with concentration of the competitive drug.

$$t_{0.2} = t'_{0.2} \times \frac{[\text{peroxidase}]}{[\text{peroxidase}]_0} \quad (2)$$

The value of b/b_0 was plotted on the ordinate against the drug concentration, D , on the abscissa. When a straight line is obtained with an intercept of 1 on the ordinate, the binding constant of the drug, K , to the bilirubin-binding site can be described by the following expression.

$$\frac{b}{b_0} = KD + 1 \quad (3)$$

The slope of the line is K .

Control experiments without albumin were performed at a bilirubin concentration of $1.2 \mu\text{M}$ (peroxidase $24 \mu\text{M}$, 5 cm optical cell) to test the effect of the drug on enzyme activity, the formation of bilirubin–drug complex or the oxidation of the drug by hydrogen peroxide and peroxidase. The effects of organic solvents were also checked; such effects were undetectable.

Results

Changes of the equilibrium concentration of unbound bilirubin on addition of various drugs were measured in terms of the rate of oxidation with hydrogen peroxide and peroxidase. The initial oxidation rate of bilirubin is proportional to the concentration of free bilirubin. Accordingly, the ratio of time periods with and without drugs ($t_{0.2(0)}/t_{0.2}$) is equal to the inverse ratio of initial velocities. This facilitates comparison of the change of concentration of free bilirubin displaced by various drugs.

The displacement of bilirubin by phenylbutazone analogues such as phenylbutazone, oxyphenbutazone, sulfipyrazone and ketophenylbutazone, is depicted in Fig. 1. The b/b_0 value increased with increasing concentration of these drugs. Similar results were also obtained from warfarin, tolbutamide, furosemide, sulfisoxazole, salicylic acid and glibenclamide, as shown in Fig. 2. However, there were large differences in the degree of displacement of bilirubin by these drugs. The displacement effect of oxyphenbutazone was the most potent. Further, the change of slope of the lines indicated that the ability of a drug to displace bilirubin was not equivalent at low and high molar ratios. Bilirubin was displaced more effectively by phenylbutazone and sulfipyrazone at low molar ratio than at high. In contrast, oxyphenbutazone, glibenclamide, warfarin, furosemide and tolbutamide displaced bilirubin more strongly at high molar ratio than at low molar ratio.

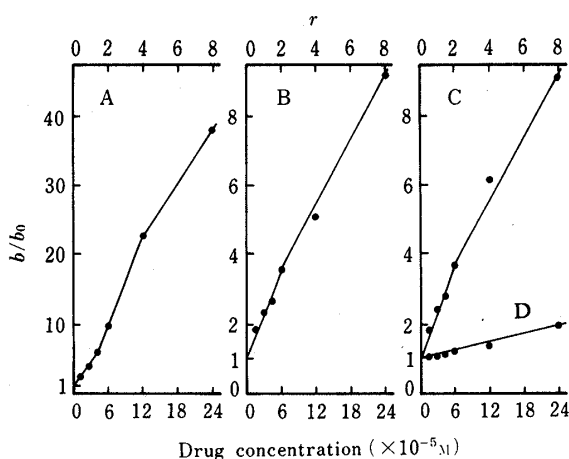


Fig. 1. Relative Concentration of Free Bilirubin in HSA–Bilirubin Complex with and without Drug as a Function of Drug Concentration

The value of b/b_0 corresponds to v/v_0 according to Eq. (1), and r is the molar ratio of drug to HSA. The values are means of 3 to 5 runs. HSA concentration was $3 \times 10^{-5} \text{M}$. The molar ratio of bilirubin to HSA is 0.6. (A) oxyphenbutazone; (B) phenylbutazone; (C) sulfipyrazone; (D) ketophenylbutazone.

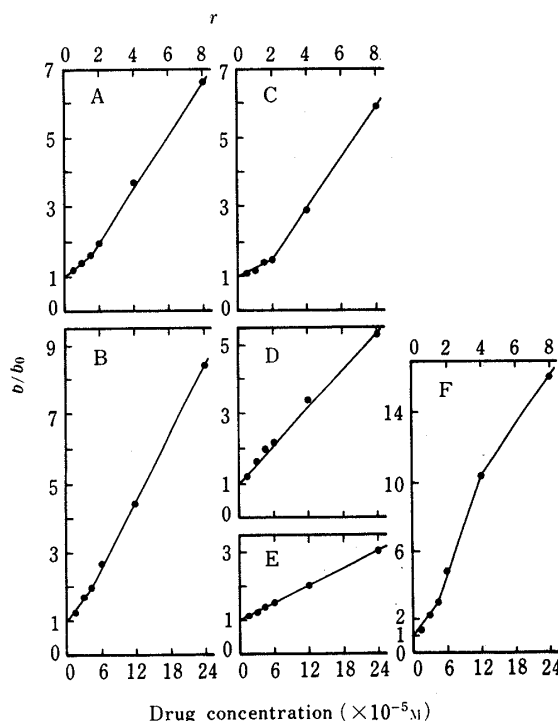


Fig. 2. Relative Concentration of Free Bilirubin in HSA–Bilirubin Complex with and without Drug as a Function of Drug Concentration

(A) warfarin; (B) tolbutamide; (C) furosemide; (D) sulfisoxazole; (E) salicylic acid; (F) glibenclamide.

TABLE I. Binding Constants of Drugs to the Bilirubin-Site of HSA^{a)}

Drug	K^b ($\times 10^4 \text{ M}^{-1}$)
Oxyphenbutazone	9.5
Sulfinpyrazone	4.8
Phenylbutazone	4.7
Glibenclamide	4.3
Tolbutamide	2.3
Sulfisoxazole	2.0
Warfarin	1.3
Salicylic acid	0.8
Furosemide	0.7
Ketophenylbutazone	0.3

- a) The data at low molar ratio of drug to albumin ($r < 1.5$) were used for calculation of the binding constant. The relationship of free bilirubin concentration to drug concentration was linear (Figs. 1 and 2).
 b) Calculated by Eq. (3).

As shown in Figs. 1 and 2, a linear relationship was obtained between drug concentration and free bilirubin concentration at low molar ratio of drug to albumin. The increase in the amount of free bilirubin at 0.5—1.5:1 ratio of drug to albumin may be ascribed to competitive binding of the drug to the bilirubin-binding site. The slope of the line was equal to the binding constant (K) of the drug for the high-affinity site of bilirubin. The calculated K values are summarized in Table I. However, this linear relationship was not always maintained at excess drug concentration. Complicated interactions may occur in the secondary binding, possibly involving an allosteric mechanism or competition. Among phenylbutazone analogues, the K value of oxyphenbutazone was twice as large as that of phenylbutazone. These results indicate that the primary binding site of these drugs may be identical with or adjacent to the bilirubin-binding site on the HSA molecule.

On the other hand, as shown in Fig. 3, flufenamic acid, mefenamic acid, ibuprofen, ethacrynic acid and acetohexamide produced a different type of displacement of bilirubin compared with the drugs mentioned above. At relatively low drug concentration (up to a ratio of about 1.5 on the abscissa) the free bilirubin concentration was not changed by these drugs. These results indicate that these drugs may bind to a different site from that of bilirubin. However, at about 2-fold molar excess of these drugs over albumin, bilirubin was significantly displaced, suggesting that the drugs under these conditions can interact with the binding site of bilirubin. It is suggested that at least the primary binding site of these drugs is not identical with that of bilirubin.

Clofibrate, buformin and phenytoin were clearly different from the two types mentioned above. As shown in Fig. 4, these drugs did not displace bilirubin at a molar ratio of 8, suggesting that the primary and secondary binding sites of these drugs do not interact with the bilirubin-binding site.

Discussion

There is increasing evidence that drug-binding sites exist on the HSA molecule and interest has recently been focused on the specificity of these sites. Such knowledge is obviously necessary to prove or predict drug interactions at the protein binding level and to predict effects of the protein binding of a drug on disease states of patients. The specific binding sites

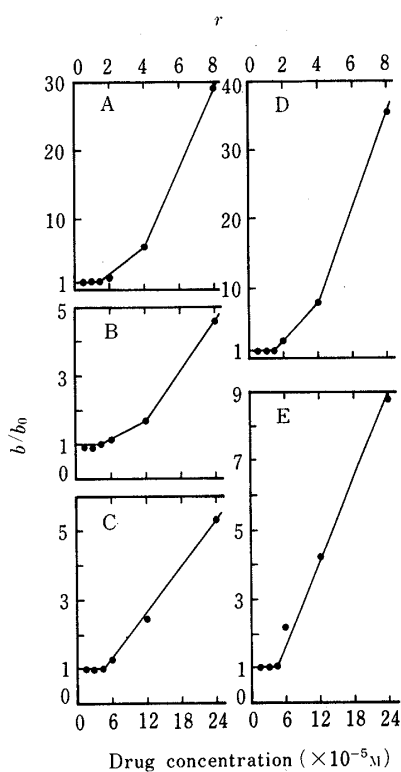


Fig. 3. Relative Concentration of Free Bilirubin in HSA-Bilirubin Complex with and without Drug as a Function of Drug Concentration

(A) flufenamic acid; (B) ibuprofen; (C) ethacrynic acid; (D) mefenamic acid; (E) acetohexamide.

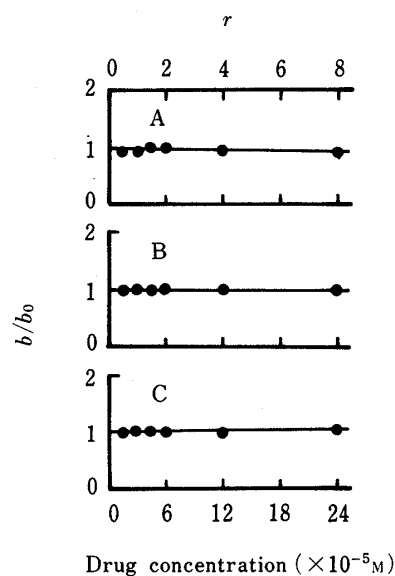


Fig. 4. Relative Concentration of Free Bilirubin in HSA-Bilirubin Complex with and without Drug as a Function of Drug Concentration

(A) clofibrate; (B) buformin; (C) phenytoin.

of drugs have been studied by various methods. Data accumulated to date suggest that the actual number of high-affinity sites of the HSA molecule for drugs is rather small; two or three. The high-affinity binding site(s) for coumarin anticoagulants, phenylbutazone analogues and sulfisoxazole is considered to be identical and has been called Site I,⁵⁾ the U-site⁸⁾ and the warfarin-site.¹⁸⁾ On the other hand, that for ibuprofen, ethacrynic acid, flufenamic acid and diazepam is clearly different from the above sites, and is called Site II,⁵⁾ the R-site⁸⁾ and the diazepam-site.¹⁸⁾

The purpose of the present study was to evaluate the displacement of bilirubin by various drugs at low molar ratio of drug to HSA through kinetic measurements of the free bilirubin concentration by means of enzymatic oxidation. It has been observed that bilirubin binds to HSA with one high-affinity site and one or two low-affinity sites.¹⁵⁾ Jacobsen^{12,13)} has obtained evidence that Lys-240 in HSA is involved in the high-affinity binding site of bilirubin (bilirubin-site), based on the results of covalent coupling of bilirubin to HSA with carbodiimide. Further, it was confirmed that the location of the bilirubin-site is on part of loop 4 (containing Lys-240) in the model of the secondary structure of HSA.^{19,20)} Therefore, bilirubin is a suitable marker for the characterization of drug-binding sites on albumin.

As shown in Figs. 1 and 2, phenylbutazone, oxyphenbutazone and warfarin bound to Site I⁵⁾ showed similar displacement effects on bilirubin, and all drugs of this type were classified as group I drugs. However, there were large differences in the degree of displacement among drugs. On the other hand, ibuprofen, flufenamic acid and ethacrynic acid, bound to Site II,⁵⁾ showed similar displacement, but were different from group I drugs. Mefenamic acid and acetohexamide were also of this type (Fig. 3). These drugs were, therefore, classified as

TABLE II. Classification of Drugs on the Basis of the Primary Binding Site

Group I	Group II	Group III
Phenylbutazone ^{a)}	Flufenamic acid ^{b)}	Clofibrate
Oxyphenbutazone ^{a)}	Mefenamic acid	Buformine
Sulfinpyrazone ^{a)}	Ibuprofen ^{b)}	Phenytoin
Ketophenylbutazone	Acetohexamide	
Warfarin ^{a)}	Ethacrynic acid ^{b)}	
Tolbutamide		
Glibenclamide		
Salicylic acid		
Furosemide		
Sulfisoxazole		

a) and b) are classified into Site I and Site II by Sudlow *et al.* (ref. 5).

group II drugs. Clofibrate, buformine and phenytoin could not displace bilirubin, and are different from the above drugs. Therefore these drugs were classified as group III drugs (Fig. 4). These results are summarized in Table II. Though tolbutamide and glibenclamide are group I drugs, they were shown to bind to both the diazepam-site and warfarin-site by Sjöholm *et al.*,¹⁸⁾ and tolbutamide was among the R-site drugs reported by Ozeki *et al.*⁸⁾ Further, phenytoin was suggested to bind to the warfarin-site.¹⁸⁾ However, a relationship between phenytoin and the warfarin-site can be ruled out, since the present study showed that phenytoin did not replace bilirubin. Thus, the situation is not straightforward.

Group I drugs at low molar ratio of drug to albumin gave a linear relationship between free bilirubin concentration and drug concentration (Figs. 1 and 2). Because bilirubin was bound tightly to the bilirubin-site on the HSA molecule under the present experimental conditions, it was concluded that the group I drugs decreased the binding of bilirubin to the bilirubin-site on albumin. The results suggest that the binding site of bilirubin and that of the group I drugs are the same. The increase of free bilirubin concentration may be described in terms of competitive binding of the group I drugs to the bilirubin-site. The K value obtained from the slope of the line may reflect not only the binding affinity for the bilirubin-site but also the interaction between the bilirubin-site and the drug binding site. It was reported that the high-affinity binding site of warfarin is strongly affected by chemical modification of the lone tryptophan residue of HSA, while that of bilirubin, phenylbutazone and oxyphenbutazone is not.²¹⁻²³⁾ Therefore, the warfarin-site could be distinct from the binding sites of bilirubin, phenylbutazone and oxyphenbutazone. However, the lone tryptophan residue of HSA at position 214 and the lysine residue at 240 are located fairly close to each other within the primary structure of HSA, so that interaction between the warfarin-site and the bilirubin-site may be possible, depending on the conformation of the albumin molecule. The results in Table I suggested that the binding site of oxyphenbutazone was most adjacent to the bilirubin-site while that of ketophenylbutazone was the most distant from it. Thus it can be assumed that the primary site of group I drugs has a large binding area overlapping the bilirubin-site. This site may be in the neighborhood of the lone tryptophan residue and the hydrophobic area corresponding to loops 3—4 in the model of the secondary structure of HSA. Loop 3 (adjacent to loop 4) is rich in hydrophobic groups in spite of the small peptide.¹⁴⁾ Fragment C isolated by CNBr degradation contains loops 3 and 4.²⁴⁾ Gambhir *et al.*⁶⁾ supposed that Fragment C is the major locus for ligand binding in HSA. The displacement effect at high molar ratio of drug to albumin suggested that the secondary binding site of group I drugs also overlaps the area of loops 3—4. However, the situation is

complex for some drugs. Because the secondary binding of drugs is of low affinity and has a large number of binding sites in general, displacement effects may involve complicated interactions, such as conformational change of albumin caused by allosteric effects.

The primary binding of the group II and the group III drugs did not displace bilirubin from its site. However, the group II drugs displaced bilirubin when the molar ratio of drug to albumin was more than 1.5, while the group III drugs did not (Figs. 3 and 4). These observations suggest that the primary binding site of the group II drugs does not overlap the bilirubin-site, but the secondary site does. Therefore, it was concluded that the primary binding site of the group II drugs is independent of the secondary site. Brodersen¹⁰⁾ found that benzodiazepine-binding sites were clearly independent of the bilirubin-site on HSA. Sjöholm *et al.*¹⁸⁾ reported that the binding sites of ethacrynic acid and ibuprofen could be classified as the diazepam-site. From their observations and the present results, it can be concluded that the primary binding sites of the group II drugs are identical with the diazepam-site. However, the location of this site is not yet clear.

The group III drugs did not displace bilirubin in primary or secondary binding, and so the binding site of the group III drugs seems to be completely independent of the bilirubin-site.

In the previous report, the classification of the drugs into three different groups was deduced, but the present classification is not directly comparable to the previous one because the measurements at low molar ratio of drug to albumin can exclude complications which accompanied previous measurements such as secondary and tertiary binding effects.

Thus, it was shown that there are three different categories in the displacement of bilirubin by drugs, and the drugs could be classified into three groups, I, II and III.

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