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Structural Requirements for Drug Binding to Site II on Human Serum Albumin

SOMPON WANWIMOLRUK[1], DONALD J. BIRKETT, AND PETER M. BROOKS[2]

Departments of Clinical Pharmacology and Medicine, The Flinders University of South Australia, Adelaide, South Australia 5042, Australia

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SUMMARY

Structure-activity relationships for binding at site II on human serum albumin have been investigated using drugs, fatty acids, and aliphatic amines with chain lengths C-3 to C-18. A negative charge is not required for binding provided a strongly electronegative center is present. For example, diazepam, a basic drug that exists mainly in the un-ionized form at neutral pH, also binds with high affinity to site II. However, aliphatic amines (pK_a values 10–11) with chain lengths C-3 to C-12 did not displace markers from either site I or II, showing that the presence of a positive charge precludes binding at these sites. Short-chain fatty acids, C-3 to C-5, did not displace marker drugs or fluorescent probes from either site I or II when added at equimolar ratios with albumin. Displacement of site II (but not site I) markers occurred with medium-chain fatty acids, C-7 to C-11, and was maximal at C-10. Fatty acids with chain lengths C-10 to C-18 caused an enhancement of fluorescence of dansylamide bound to site I, the maximal effect occurring with C-12. Both arylpropionic acid nonsteroidal anti-inflammatory drugs and medium-chain fatty acids binding at site II had molecular lengths within the range 11–16 Å. The effect of hydrophobicity (and/or molecular length) on binding affinity was much more marked with the arylpropionic acids than with the fatty acids, suggesting that bulkier aromatic molecules form more effective interactions at the binding site. The results suggest that site II is a hydrophobic cleft about 16 Å deep and about 8 Å wide in the albumin molecule with a cationic group located near the surface.

INTRODUCTION

HSA[3] is the most important binding protein in plasma for drugs and endogenous substances, and its ready availability has greatly helped to gain insight into the mechanisms of ligand protein interactions. The displacement of one drug by another from albumin binding sites contributes to drug interaction phenomena (1–4), and drug binding is also altered in a number of disease states, particularly hepatic and renal diseases. These changes may affect the distribution and elimination of drugs.

There is considerable evidence that only a few specific drug-binding sites are present on HSA. The most important are sites I and II, which are also called the warfarin-binding site, and the indole- or benzodiazepine-binding site, respectively (5–7). Site I is the primary binding site for drugs like warfarin and phenylbutazone analogues, whereas diazepam and arylpropionic acid NSAID such as ibuprofen and flurbiprofen are bound primarily to site II. It has been suggested that drugs which bind to site I are bulky heterocyclic molecules with a negative charge localized in the middle of the molecule and that site II drugs are aromatic carboxylic acids with a generally extended shape carrying the negative charge on the carboxyl group at one end of the molecule away from the hydrophobic center (5). Like site II drugs, fatty acids are extended hydrophobic molecules with a carboxyl group at one end. Therefore, we have used fatty acids and aliphatic amines with chain lengths C-3 (propionic acid) to C-18 (stearic acid) as well as some NSAID to define further the characteristics and structural requirements for drug binding at site II on HSA.

EXPERIMENTAL PROCEDURES

Materials. HSA, essentially fatty acid-free (Lot No. 110F-9350), DNSA, dansylsarcosine, and the sodium salts of palmitic acid and stearic acid were purchased from Sigma Chemical Company (St. Louis, Mo.). The essentially fatty acid-free HSA contained 0.04 mole of fatty acid per mole of albumin, as determined by the method of Duncombe

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[1] Present address, Department of Pharmacology, Faculty of Science, Prince of Songkla University, Haad-Yai, Thailand.

[2] Department of Medicine, The Flinders University of South Australia.

[3] The abbreviations used are: HSA, human serum albumin; NSAID, nonsteroidal anti-inflammatory drugs; DNSA, 5-dimethylaminonaphthalene-1-sulfonamide; GP53,633, 2-*tert*-butyl-4(5)-phenyl-5(4)-(3-pyridyl)-imidazole.

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(8). The molecular weight of albumin was taken as 66,500 (9). All other chemicals and compounds used were of analytical grade and were obtained from the manufacturers or from commercial suppliers. ^{14}C-Labeled warfarin was purchased from the Radiochemical Centre (Amersham, United Kingdom). [^{14}C]GP53,633 (CIBA-Geigy Ltd.), [^{14}C] ibuprofen, and [^{14}C]flurbiprofen (Boots Company Ltd., Sydney, Australia) were kindly donated by the manufacturers. The radiochemical purities of these compounds as stated by the suppliers were greater than 99%. In each case, the radiochemical purity of free drug (on the buffer side after equilibrium dialysis against human serum) was better than 96%.

Binding experiments. Fluorescent probe techniques were used to study the displacement of marker probes binding to specific binding sites on HSA as described previously by Sudlow *et al.* (10). The coefficient of variation for this technique was 2.1%. Fluorescence was measured with a Perkin-Elmer MP-3000 fluorescence spectrometer at 23°. Equilibrium dialysis experiments were performed with a Dianorm apparatus at 37° as described earlier (11). The average coefficient of variation for dialysis measurement of free fraction was 2.7%. Each measurement was performed in duplicate. All experiments were performed using sodium phosphate buffer (0.1 M, pH 7.4) with 0.9% NaCl. When necessary, the compounds were dissolved initially in a small volume of 0.1 M NaOH or 0.1 M HCl, but the final pH of all solutions was in the range 7.2–7.6; the addition of small aliquots did not affect buffer pH. The sodium salts of palmitic acid and stearic acid were evenly dispersed in the buffer by sonication and then incubated with HSA at 37° to obtain a clear solution.

Partition coefficient determination. The *n*-octanol/water partition coefficients of arylpropionic acid NSAID were determined by a flask-shaking method (12), using 0.2 M KCl/HCl (pH 1.1). After partitioning and centrifugation, a sample of the aqueous layer (80 μl) was analyzed by high-performance liquid chromatography using a modification of a method for the determination of aspirin (13). The mobile phase consisted of acetonitrile and 0.03% phosphoric acid (pH 2.5) (40:60). Absorbance of the eluent was monitored at a wavelength of 254 nm. The concentration of drugs in the water layer was determined by comparison of the peak height with a calibration curve for each drug.

Molecular measurements. The length and width of compounds were measured by a structural scale model (CATALIN Ltd., London).

RESULTS

Binding of fatty acids (C-3 to C-18) to sites I and II on HSA. Fatty acids with chain length from C-3 to C-18 were investigated to determine their specificity of binding to sites I and II. DNSA and dansylsarcosine were used as fluorescent markers for sites I and II, respectively (10). Warfarin and GP53,633 (14) were used as markers for site I, and ibuprofen and flurbiprofen as markers for site II in the equilibrium dialysis technique. Changes in the fluorescence of the probes and in the free fractions of the drugs provided a measure of displacement by fatty acids at the two sites. Figure 1 shows the changes in the fluorescence of DNSA or dansylsarcosine bound to HSA on the addition of fatty acids at a 1:1 ratio of fatty acid to HSA. Short-chain fatty acids, C-3 to C-5, did not displace dansylsarcosine, whereas medium chain fatty acids, C-6 to C-12, caused marked displacement with a maximal effect by the C-10 acid. Neither long-chain fatty acid, C-16 or C-18, displaced dansylsarcosine. No change in the fluorescence of DNSA was observed on addition of the C-3 to C-9 acids. However, DNSA fluorescence was increased by the longer-chain acids, C-10 to C-18.

In agreement with the fluorescent probe techniques, results with equilibrium dialysis showed that the free fractions of the site II markers, ibuprofen and flurbiprofen, were markedly increased only by addition of medium-chain fatty acids, C-6 to C-12 (Table 1). Fatty acids C-3 to C-10 caused no change in the free fractions of site I markers, warfarin and GP53,633. On the other hand, the long-chain fatty acids, C-11 to C-18, caused a decrease in the free fractions of both warfarin and GP53,633 (i.e., increased the binding).

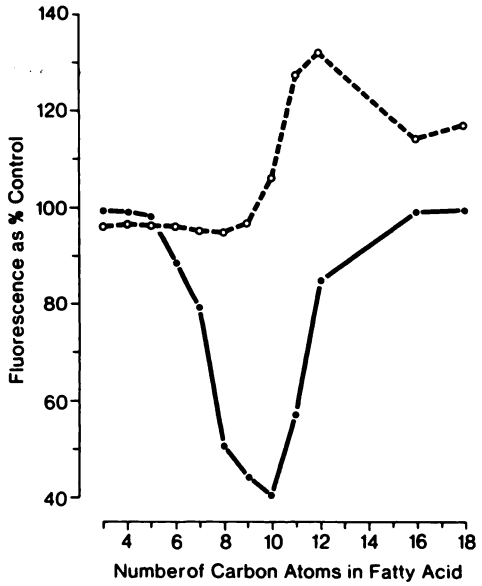


FIG. 1. *Effect of fatty acids (C-3 to C-18) on fluorescence of DNSA (○--○) and dansylsarcosine (●—●) bound to HSA*

The fluorescence of solutions containing 20 μM HSA and 2 μM DNSA or 2 μM dansylsarcosine was measured (excitation 350 nm, emission 475 nm) before and after the addition of fatty acids at a 1:1 molar concentration ratio to albumin. Values represent the fluorescence of the probes bound to HSA in the presence of fatty acids as a percentage of the fluorescence of the probes bound to HSA alone.

TABLE 1

Displacement of marker drugs for sites I and II as measured by equilibrium dialysis

Essentially fatty acid-free HSA (100 μM) was dialyzed against an equal volume (1 ml) of buffer containing 10 μM warfarin, GP53,633, ibuprofen, or flurbiprofen with or without the addition of fatty acid (100 μM). The free fractions of the marker drugs in the absence of fatty acids were 0.071 (warfarin), 0.175 (GP53,633), 0.028 (ibuprofen), and 0.008 (flurbiprofen).

Fatty acid	Free fraction as % control			
	Site I		Site II	
	Warfarin	GP53,633	Ibuprofen	Flurbiprofen
C-3	99	100	101	96
C-4	101	99	99	103
C-5	103	102	103	99
C-6	104	99	119	119
C-7	100	94	146	148
C-8	97	88	239	255
C-9	96	72	282	304
C-10	92	51	301	341
C-11	80	27	226	236
C-12	84	27	115	111
C-16	85	65	100	101
C-18	75	46	103	101

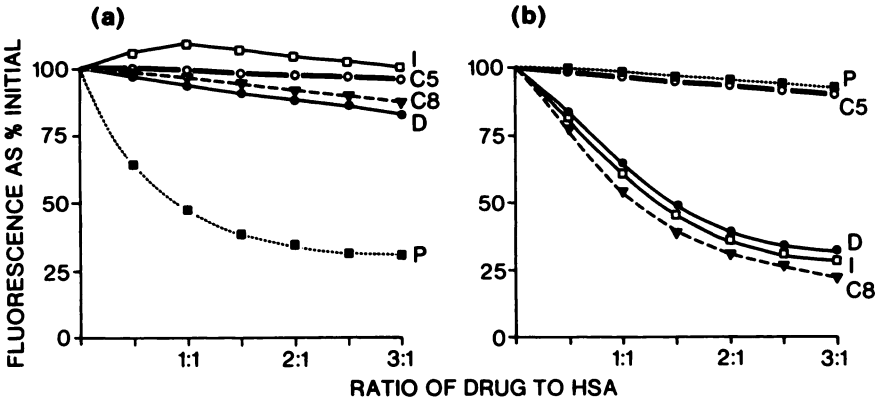


FIG. 2. *Effects of increasing concentrations of fatty acids and drugs on site I and II fluroescent probes*
The fluorescence of DNSA (a) or dansylsarcosine (b) was measured before and after the addition of fatty acids or drugs. Concentrations of probes and HSA were as in Fig. 1. The displacing compounds used were ibuprofen (*I*), phenylbutazone (*P*), pentanoic acid (*C5*), octanoic acid (*C8*), and diazepam (*D*).

The effects of higher concentrations of medium-chain fatty acids on sites I and II were investigated by fluorescence with the C-5 and C-8 acids (Fig. 2). For comparison, phenylbutazone and ibuprofen were used as displacing drugs for sites I and II, respectively. The effects of diazepam are also shown in Fig. 2. Even at molar concentration ratios up to 3:1 with HSA, pentanoic acid (C-5) had no effect at either site, and octanoic acid (C-8) displaced only the site II fluorescent marker (dansylsarcosine).

Effects of aliphatic amines at sites I and II on HSA. The addition of aliphatic amines (C-3 to C-12) up to a molar ratio of 3:1 with HSA (100 μM) caused no change in the fluorescence of either DNSA or dansylsarcosine. Free fractions of both site I markers (warfarin and GP53,633) and site II markers (ibuprofen and flurbiprofen) were not altered in the presence of these amines at molar concentration ratios of 1:1 with HSA (100 μM).

Displacement by arylpropionic acid NSAID at sites I and II. The effects of six arylpropionic acid NSAID on the fluorescence of DNSA and dansylsarcosine are shown in Fig. 3. All of these drugs were bound selectively to site II on HSA, as all of them caused a large decrease in the fluorescence of dansylsarcosine but not DNSA. Some, in fact, enhanced DNSA fluorescence. Dialysis experiments showed similar results (Table 2). When 100 μM HSA was used and drugs were added at equimolar concentrations with albumin, ibuprofen and flurbiprofen free fractions were increased in the presence of all of the arylpropionic acid derivatives studied. By contrast, the binding of the site I markers, warfarin and GP53,633, was not decreased, and was sometimes enhanced, by addition of these drugs.

Effects of diazepam on binding sites I and II. Diazepam, up to a 3:1 molar concentration ratio with HSA, did not alter the fluorescence of albumin-bound DNSA, whereas it markedly decreased the fluorescence of dansylsarcosine (Fig. 2). Warfarin and GP53,633 free fractions were not altered or were slightly decreased by addition of diazepam at a 1:1 concentration ratio with albumin (Table 2). In contrast, there was a significant increase in the free fractions of both site II marker drugs, ibuprofen and flurbiprofen, in the presence of diazepam.

Nature of dansylsarcosine displacement. The mechanism of dansylsarcosine displacement was investigated

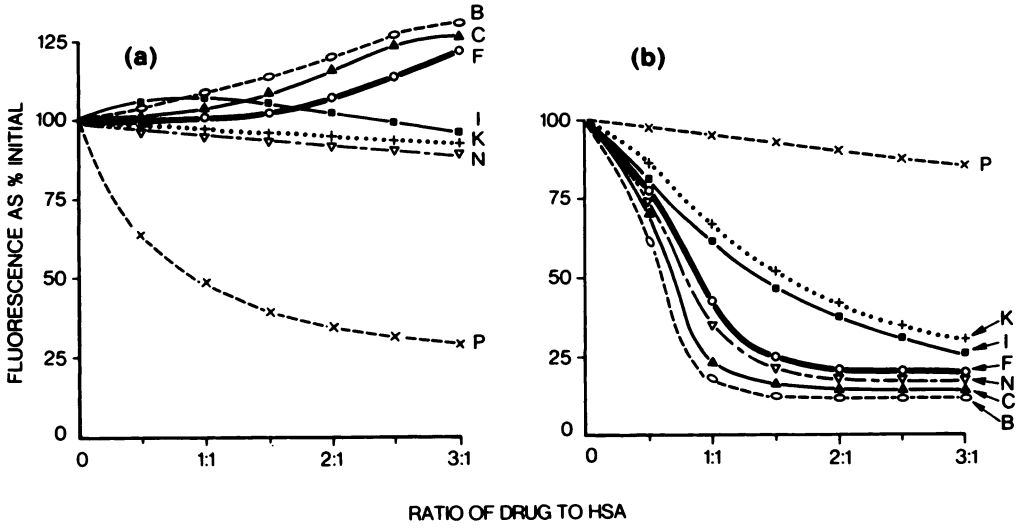


FIG. 3. *Effects of arylpropionic acid NSAID and phenylbutazone at sites I and II*
The fluorescence of DNSA (a) or dansylsarcosine (b) was measured before and after the addition of drugs. Conditions were as in Fig. 1. The drugs used were benoxaprofen (*B*), cicloprofen (*C*), flurbiprofen (*F*), ibuprofen (*I*), ketoprofen (*K*), naproxen (*N*), and phenylbutazone (*P*).

TABLE 2

Displacement of site I and site II marker drugs by arylpropionic acid NSAID and diazepam

HSA (100 μM) was dialyzed against an equal volume (1 ml) of buffer containing warfarin, GP53,633, ibuprofen, or flurbiprofen (10 μM) with or without the addition of the other drugs (100 μM). The free fractions of marker drugs in the absence of other drugs were 0.071 (warfarin), 0.168 (GP53,633), 0.026 (ibuprofen), and 0.007 (flurbiprofen).

Drug	Free fraction as % control			
	Site I		Site II	
	Warfarin	GP53,633	Ibuprofen	Flurbiprofen
Benoxaprofen	82	53	937	1060
Cicloprofen	88	71	472	585
Flurbiprofen	88	53	312	—
Ibuprofen	95	54	—	169
Ketoprofen	98	75	199	194
Naproxen	94	85	396	467
Diazepam	85	99	210	251

by the method of Hughes and Klotz (15). The results are summarized in Table 3. All displacing compounds caused an increase in the dissociation constant (k_d) for dansylsarcosine with no change in the number of binding sites (n), showing that the displacement is competitive in nature.

Correlations between ligand molecular length and binding. Tables 4 and 5 show the structure, molecular lengths, and pK_a values for the ligands studied. The molecular lengths given are the maximal lengths for the molecules measured from the carboxyl group in each case or from the primary amino group in the case of the aliphatic amines. The short-chain fatty acids which displaced site II markers (C-6 to C-11) have molecular lengths ranging from 11.0 Å (C-6) to 17.2 Å (C-11). The arylpropionic acid NSAID binding to site II also have molecular lengths within this range (from 12.2 Å for ibuprofen to 14.7 Å for benoxaprofen). Fatty acids with molecular lengths outside this range did not show any site II binding. Both the fatty acids and the arylpropionic acid derivatives have pK_a values around pH 3–5 and therefore have a negative charge at pH 7.4. The aliphatic amines have pK_a values around pH 10.6 and carry a positive charge at physiological pH, and even for those with molecular lengths in the critical range there was no evidence for binding at site II. Diazepam is a base with a pK_a of 3.3 and is therefore uncharged at pH 7.4. The maximal length of the diazepam molecule was found to be 11.6 Å, which is within the size range for fatty acids and arylpropionic acid derivatives binding to site II.

Correlations between partition coefficient and binding. There were good correlations (Fig. 4) between log partition coefficient (log P) and both displacement of dansylsarcosine (measured by fluorescence) and displacement of ibuprofen (measured by equilibrium dialysis) for the medium-chain acids and the arylpropionic acid NSAID that bind to site II. Equally good correlations were apparent between molecular length and displacement of dansylsarcosine and ibuprofen due to the high

TABLE 3

Effect of displacing compounds on binding parameters for dansylsarcosine

Dansylsarcosine (2 μM) was titrated with albumin (0–100 μM) to determine the limiting fluorescence (F_L) of dansylsarcosine when completely bound. HSA (5 μM) was then titrated with dansylsarcosine (0.5–15 μM) in the absence and presence of various displacing compounds (5 μM), and the fluorescence at each dansylsarcosine concentration (F_I) was measured. The concentration of bound dansylsarcosine (micromolar) at each point was calculated as $2F_I/F_L$. The data were then analyzed according to the method of Hughes and Klotz (15). n is the number of binding sites per mole of albumin, and K_d is the dissociation constant. Excitation was at 350 nm, and the fluorescence was read at 475 nm.

	n	K_d
		μM
Dansylsarcosine alone	1.0	3.2
+ Benoxaprofen	1.0	63.4
+ Cicloprofen	1.1	52.1
+ Flurbiprofen	0.9	29.8
+ Ibuprofen	1.1	11.6
+ Ketoprofen	0.9	7.8
+ Naproxen	1.0	40.2
+ Decanoic acid (C-10)	1.0	23.5

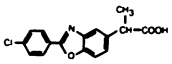
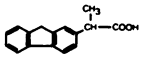
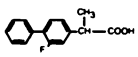
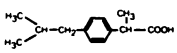
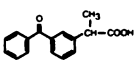
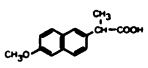
TABLE 4

Comparison of molecular lengths of fatty acids and aliphatic amines

No. of C atoms	Molecular length	
	Fatty acids (pK_a 4.9)	Aliphatic amines (pK_a 10.6)
	Å	Å
C-3	7.2	7.2
C-4	8.5	8.5
C-5	9.6	—
C-6	11.0	11.0
C-7	12.2	—
C-8	13.5	—
C-9	14.7	14.7
C-10	16.0	—
C-11	17.2	—
C-12	18.5	18.4
C-16	21.0	—
C-18	23.5	—

TABLE 5

Structures, molecular lengths, and pK_a values of arylpropionic acid NSAID

Drug	Structure	Length (Å)	pK_a
Benoxaprofen		14.7	3-4
Cicloprofen		13.2	3.70
Flurbiprofen		13.5	4.13
Ibuprofen		12.2	4.13
Ketoprofen		12.4	4.00
Naproxen		12.8	4.15

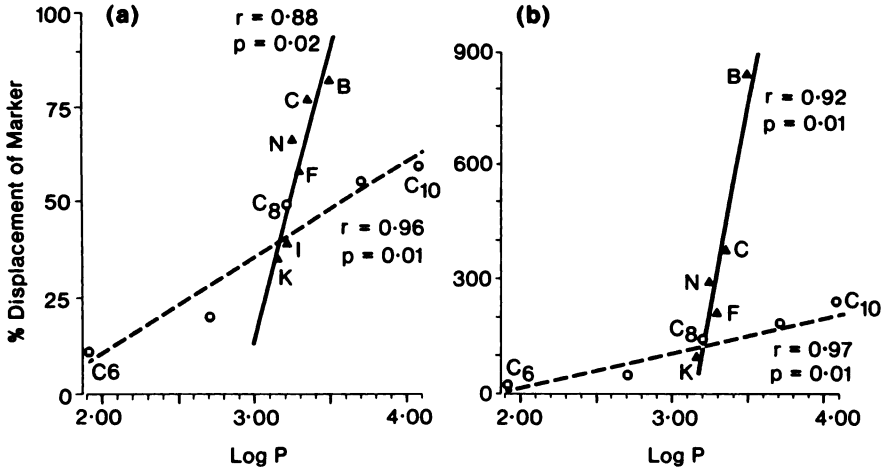


FIG. 4. *Correlation between ability to displace site II markers and partition coefficient*

Percentage displacement of dansylsarcosine (a) was measured by fluroescence and that of ibuprofen (b) was measured by dialysis. Fatty acids (O---O) are C-6 to C-10. The arylpropionic acids (▲——▲) shown are benoxaprofen (*B*), cicloprofen (*C*), naproxen (*N*), flurbiprofen (*F*), ibuprofen (*I*), and ketoprofen (*K*); *r* is the correlation coefficient derived by simple linear regression. Partition coefficient values for fatty acids were obtained from ref. 12.

degree of correlation between molecular length and log P for both fatty acids (log P = −2.68 + 0.43 length; r = 0.99) and arylpropionic acids (log P = 1.74 + 0.12 length; r = 0.95).

DISCUSSION

Although site II on HSA has been relatively well characterized with regard to location within the primary structure of HSA (16–22), the detailed structural requirements for binding with high affinity at this site have not been elucidated. The present study was suggested by the previous observations (19, 23, 24) that both medium-chain fatty acids and some site II-specific drugs inhibited the acetylation of albumin by *p*-nitrophenyl acetate, which was assumed to occur at or close to site II.

It has been proposed previously that compounds which bind at site II on albumin are aromatic carboxylic acids that are ionized at physiological pH and have extended structures with the negative charge at one end (5). The present study shows that an aromatic structure is not necessary for binding, as aliphatic carboxylic acids with appropriate chain lengths (C-6 to C-11) competitively displaced site II-specific fluorescent probes and marker drugs. By contrast, the binding of fluorescent probes and drugs at site I was unaffected, indicating that the medium-chain fatty acids bind selectively at site II under the conditions used.

Molecular interactions are determined fundamentally by molecular size, shape, and charge distribution (25). In the present study, the molecular lengths of compounds binding at site II have been measured using models giving standard bond lengths and angles. The values obtained from the models were consistent with estimates by crystallographic coordinate methods where crystal structures were available. For example, the molecular model of flurbiprofen gave a length of 13.5 Å, and the value calculated from crystallographic co-ordinates (26) was 13.4 Å. The models for the aliphatic acids and amines were constructed in the zigzag conformation, and those for the arylpropionic acids were constructed with reference to the crystallographic structure for flurbiprofen (26). Models and molecular dimensions for pentanoic acid, octanoic acid, and flurbiprofen are illustrated in Fig. 5. These results indicate (Fig. 1 and Table 1) that only fatty acids with chain lengths C-6 to C-11 (molecular lengths 11–17.2 Å) bind at site II. Consistent with this indication was the finding that all of the arylpropionic acids binding at site II had molecular lengths within this range. Fatty acids with longer chain lengths did not bind at site II, but rather at a site which interacts, presumably by an allosteric mechanism, with site I, causing enhanced binding of drugs at this latter site. None of the fatty acids or arylpropionic acids displaced marker compounds for site I, which is consistent with the proposal that compounds binding at site I are bulky molecules with the negative charge, if present, located close to the center of the molecule (5).

The effect of charge on site II binding was investigated using aliphatic acids and amines of appropriate chain lengths (Table 4). At physiological pH, both acids and amines are ionized, with the acids having a negative charge and the amines a positive charge. The aliphatic amines, even though of appropriate chain length, did not

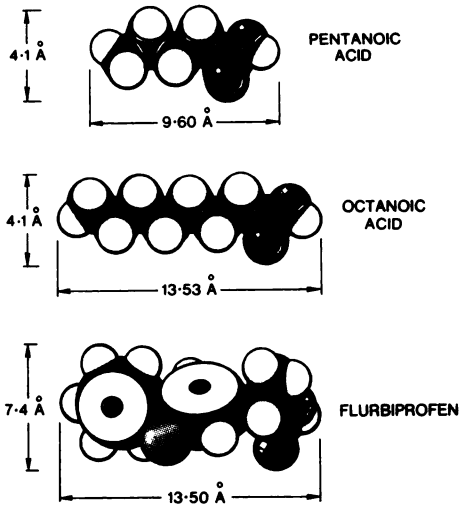


FIG. 5. *Molecular models for pentanoic acid, octanoic acid, and flurbiprofen*

displace at site II, suggesting that the presence of a positive charge precludes binding at this site. This is in accord with the observations of Chignell (27), who concluded that the binding of flufenamic acid at site II involved, as well as hydrophobic interactions, electrostatic interactions between the carboxyl group and a cationic center on the protein surface. Sjöholm found that benzodiazepines containing electronegative centers bind strongly to site II whereas those with cationic centers at physiological pH do not (28). Fehske *et al.* (21, 22) have suggested that the basic amino acid residues lysine 194 and arginine 145 are at or close to site II.

Although a positive charge precludes binding, a negative charge is not necessary. Diazepam, a base but with a low pK_a and therefore un-ionized at pH 7.4, binds strongly at site II (Table 2 and Fig. 2). The maximal length of the diazepam molecule was found to be 11.6 Å, which is within the range for the other site II compounds. Similar findings were reported by us in relation to site I. GP53,633, a basic phenylbutazone analogue, was shown to bind at site I in the un-ionized but not in the ionized form (14). Other investigators have shown correlations between partition coefficient and albumin binding affinity for penicillins (29), sulfonamides (30, 31), fenamates (27), and several homologous series of drugs (31, 32). In the present study the hydrophobicity of the un-ionized forms of both fatty acid and arylpropionic acid NSAID was highly correlated with ability to displace at site II (Fig. 4), but the effects of hydrophobicity could not be separated from those of molecular length because of the high correlation between these parameters. The increasing length of the arylpropionic acids was associated with less change in partition coefficient than for the fatty acids, presumably due to the marked contribution of the aromatic groups in the former case. By contrast, increasing hydrophobicity, and length, of the arylpropionic acid NSAID resulted in far greater increases in site II marker displacement than was the case for the aliphatic fatty acids (Fig. 4), suggesting that molecular parameters other than length, hydrophobicity, and charge are also important binding determinants. The arylpropionic acid NSAID are bulkier molecules (maximal molecular widths 6.1–7.8 Å) than the fatty acids (maximal width about 4.1 Å), which may allow more effective hydrophobic interactions with groups in the binding cleft (Fig. 5).

This study supports the view that site II is a hydrophobic cleft 12–16 Å deep and 6–8 Å wide with a cationic group located near the surface of the protein. The cationic center precludes the binding of ligands carrying a positive charge, but ligands with a negative charge or strongly electronegative center are able to bind at this site. Although hydrophobic forces appear to provide the main binding energy, the relationship between hydrophobicity and binding is complex and depends on other features, such as molecular dimensions of ligands binding at this site.

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REFERENCES

1. Koch-Weser, J., and E. M. Sellers. Binding of drugs to serum albumin. *N. Engl. J. Med.* **294**:311–316 (1976).
2. Powell-Jackson, P. R. Interaction between azapropazone and warfarin. *Br. Med. J.* **1**:1193–1194 (1977).
3. Bjornsson, T. D., P. J. Meffin, S. Swezey, and T. F. Blaschke. Clofibrate displaces warfarin from plasma proteins in man: an example of a pure displacement interaction. *J. Pharmacol. Exp. Ther.* **210**:316–321 (1979).
4. Tillement, J. P., F. Lhoste, and J. F. Guidicelli. Diseases and drug protein binding. *Clin. Pharmacokinet.* **3**:144–154 (1978).
5. Sudlow, G., D. J. Birkett, and D. N. Wade. Further characterization of specific drug binding sites on human serum albumin. *Mol. Pharamcol.* **12**:1052–1061 (1976).
6. Birkett, D. J., S. Ray, G. Sudlow, and J. Hagedorn. Fluorescent probe studies of albumin binding sites. *Acta Pharm. Suec.* **17**:78 (1980).
7. Sjöholm, I., 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).
8. Duncombe, W. G. The colorimetric micro-determination of non-esterified fatty acids in plasma. *Clin. Chim. Acta* **9**:122–125 (1964).
9. Meloun, B., L. Moravek, and V. Kostka. Complete amino acid sequence of human serum albumin. *F. E. B. S. Lett.* **58**:134–137 (1975).
10. 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).
11. Wanwimolruk, S., D. J. Birkett, and P. M. Brooks. Protein binding of some non-steroidal anti-inflammatory drugs in rheumatoid arthritis. *Clin. Pharmacokinet.* **7**:85–92 (1982).
12. Leo, A., C. Hansch, and D. Elkins. Partition coefficients and their uses. *Chem. Rev.* **71**:525–616 (1971).
13. Rumble, R. H., M. S. Roberts, and S. Wanwimolruk. Determination of aspirin and its major metabolites in plasma by high-performance liquid chromatography without solvent extraction. *J. Chromatogr.* **225**:252–260 (1981).
14. Wanwimolruk, S., D. J. Birkett, and P. M. Brooks. Protein binding of GP53,633: a basic non-steroidal anti-inflammatory drug. *Biochem. Pharmacol.* **31**:3737–3743 (1982).
15. Hughes, T. R., and I. M. Klotz. Analysis of metal-protein complexes. *Methods Biochem. Anal.* **3**:265–299 (1956).
16. McMenamy, R. H., and J. L. Oncley. The specific binding of L-tryptophan to serum albumin. *J. Biol. Chem.* **233**:1436–1447 (1958).
17. Müller, W. E., and U. Wollert. High stereospecificity of the benzodiazepine binding site on human serum albumin: studies with *d*- and *l*-oxazepam hemisuccinate. *Mol. Pharmacol.* **11**:52–60 (1975).
18. Gambhir, K. K., R. H. McMenamy, and F. Watson. Positions in human serum albumin which involve the indole binding site. *J. Biol. Chem.* **250**:6711–6719 (1975).
19. Sollenne, N. P., and G. E. Means. Characterization of a specific drug binding site of human serum albumin. *Mol. Pharmacol.* **15**:754–757 (1979).
20. Fehske, K. J., W. E. Müller, and U. Wollert. Direct demonstration of the highly reactive tyrosine residue of human serum albumin located in fragment 299-585. *Arch. Biochem. Biophys.* **205**:217–221 (1980).
21. Fehske, K. J., W. E. Müller, U. Schläfer, and U. Wollert. Characterization of two important drug binding sites on human serum albumin, in *Progress in Drug Protein Binding* (N. Rietbrock and B. G. Woodcock, eds.). Proceedings of lectures presented at a symposium in Frankfurt am Main, April 1980. Braunschweig, Weisbaden, Vieweg 5–15 (1981).
22. Fehske, K. J., W. E. Müller, and U. Wollert. The location of drug binding sites in human serum albumin. *Biochem. Pharmacol.* **30**:687–692 (1981).
23. Koh, S. W., and G. E. Means. Characterization of a small apolar anion binding site on human serum albumin. *Arch. Biochem. Biophys.* **192**:73 (1979).
24. Ozeki, Y., Y. Kurono, T. Yotsuyanagi, and K. Ikeda. Effects of drug binding on the esterase activity of human serum albumin: inhibition modes and binding sites of anionic drugs. *Chem. Pharm. Bull.* **28**:535–540 (1980).
25. Ganellin, R. 1980 Award in Medicinal Chemistry. Medicinal chemistry and dynamic structure-activity analysis in the discovery of drugs acting at histamine H_2 receptors. *J. Med. Chem.* **24**:913–920 (1981).
26. Flippen, J., and R. D. Gilardi. (±)-2-(2-Fluoro-4-biphenyl) propionic acid (flurbiprofen). *Acta Crystallogr. Sect. B* **31**:926–928 (1975).
27. Chignell, C. F. Optical studies of drug-protein complexes. III. Interaction of flufenamic acid and other *N*-arylanthranilates with serum albumin. *Mol. Pharmacol.* **5**:455–462 (1969).
28. Sjöholm, I. Binding of drugs to human serum albumin, in *Albumin: Structure, Biosynthesis, Functions* (T. Peters, Jr., and I. Sjöholm, eds.). Federation of European Biochemical Societies 11th Meeting, Copenhagen. Pergamon Press, Oxford, 71–78 (1978).
29. Bird, A. E., and A. C. Marshall. Correlation of serum binding of penicillins with partition coefficients. *Biochem. Pharmacol.* **16**:2275–2290 (1967).
30. Fujita, T. Hydrophobic bonding of sulfonamide drugs with serum albumin. *J. Med. Chem.* **15**:1049–1056 (1972).
31. Seydel, J. K., and K. J. Schaper. Quantitative structure-pharmacokinetic relationships and drug design. *Pharmacol. Ther.* **15**:131–182 (1982).
32. Lucek, R. W., and C. B. Coutinho. The role of substituents in the hydrophobic binding of the 1,4-benzodiazepines by human plasma proteins. *Mol. Pharmacol.* **12**:612–619 (1976).

Send reprint requests to: Professor D. J. Birkett, Department of Clinical Pharmacology, Flinders Medical Centre, Bedford Park, South Australia 5042, Australia.