Biochemical Pharmacology, Vol. 38, No. 5, pp. 753–758, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

EVIDENCE FOR ISOXICAM BINDING TO SITE I AS A PRIMARY SITE AND TO SITE II AS A SECONDARY SITE OF HUMAN SERUM ALBUMIN

FRANÇOISE BREE,* PHUC NGUYEN, EDITH ALBENGRES, SAÏK URIEN, PASCALE RIANT, PETER G. WELLING† and JEAN-PAUL TILLEMENT

Laboratoire de Pharmacologie, Faculté de Médecine, 8 rue du Général Sarrail, 94010 Creteil, France; and † Warner Lambert, Ann Arbor, Michigan 48105, U.S.A.

(*Received* 1 *July* 1988; *accepted* 31 *August* 1988)

Abstract—Isoxicam binding to HSA was studied using equilibrium dialysis and fluorescence methods. It was shown that this drug binds to or near site I (warfarin or azapropazone site) and to site II (the diazepam site) as a secondary site, although it is generally considered that their respective drug structural requirements are often exclusive. The binding parameters were calculated with different mathematical models; a site oriented model with or without fixing the number of binding sites as integer values and a stoichiometric model. The relevant results are in good agreement under the selected experimental conditions. The stoichiometric method indicates that no positive cooperativity occurred during the binding process but other interactions between the two sites cannot be excluded.

Selective binding sites have been described for drugs on human serum albumin (HSA). Two of them, sites I and II [1–3] are mainly, but not exclusively, devoted to acidic ligands providing they are ionized at plasma pH: their binding involves both electrostatic and hydrophobic bonds. Site I, also called the warfarin site, binds bulky heterocyclic molecules with at least one enol group and a negative charge in the center of the largely non-polar molecule [4]. Site II, called the site for indoles and benzodiazepines, also binds arylpropionic acids and aromatic molecules having generally but not necessarily a free carboxylic acid at one end of the molecule away from the hydrophobic center [4–6]. A full negative charge is not required for a binding to this site since diazepam, unionized at pH 7.4, also binds with a high affinity to this site. The binding of compounds to site II was found to be closely related to their respective hydrophobicities [6] suggesting that hydrophobic interactions provide the main binding energy. As the structural requirements of these sites are different, it may be expected that an acidic ligand would bind either to site I or to site II but rarely to both. Nevertheless, some compounds such as sodium valproate [7], floctafenic acid [8], and some sulfonamides including tolbutamide, tolazamide and sulfadimethoxine [2] bind to both sites.

It seems of interest to document such dual binding to find out which chemical structures may be compatible with simultaneous binding, and also because such drugs may compete at both sites thus developing multiple drug interactions. It has also to be checked if the two binding processes are really independent as the two sites are relatively close in the protein structure [3].

For this purpose, we used isoxicam, a new oxicam derivative (Fig. 1), exhibiting anti-inflammatory effects both in animals and in man [9–11]. Previous

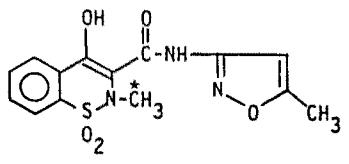

Fig. 1. Chemical structure of isoxicam. (*)^{14}C labelling.

results (unpublished data) using equilibrium dialysis data and calculations with a site specific model program [12] gave approximately 2 binding sites per mole of HSA. From its chemical structure, binding to site I was likely. Thus the question was to know if it was also bound to site II.

This was analyzed in two ways. The first one was an interpretation of equilibrium dialysis data with two different computation methods using the site specific and the stoichiometric models. The first method, derived from the Scatchard equation was used either by fixing integer values for the number of binding sites, or by allowing free, non integer values for them. The stoichiometric method [13] used derived from the stepwise equilibrium model [14]. Both results were compared and supported the occurrence of two binding sites. The two Scatchard derived methods were then used for the analysis of further experiments aimed at characterizing the isoxicam binding sites on HSA. Equilibrium dialysis methods were used to determine the alterations of binding induced by various competitors selective for either site I (warfarin, phenylbutazone and azapropazone) or site II (ibuprofen and diazepam). As the HSA–isoxicam complex develops native fluorescence, it was also possible to check the nature of the binding sites by analyzing the quenching that these selective ligands induce. Finally as palmitic

* Author for reprint requests.

and arachidonic acids increase site I binding and decreases site II binding [5], it was also used to characterize the binding sites.

The results support a simultaneous binding to both sites I and II.

MATERIALS AND METHODS

Human serum albumin. HSA (Sigma A 1887, FFA molar ratio = 0.04) was used dissolved in phosphate buffer (0.067 mM) at pH 7.4. HSA concentration was estimated by the bromocresol green method. The HSA concentration used was 20 μM.

Chemicals. Isoxicam and ^{14}C-isoxicam (1284 GBQ/mmol) were provided by Warner Lambert Company. The radiochemical purity (99.76%) was assessed by thin layer chromatography using the following solvent system: chloroform/toluene/formic acid (85/10/5; v/v). Azapropazone, ibuprofen, phenylbutazone, warfarin and diazepam were provided respectively by Siegfried SA, Boots-Dacour, Ciba-Geigy, Merrell-Toraude and Roche Laboratories. Palmitic and arachidonic acids were purchased from Sigma.

Equilibrium dialysis experiments. Drug binding was studied using equilibrium dialysis, the experiments being carried out at 37°, pH 7.4 under constant rotation at 20 rpm (Dianorm® apparatus), the volume of each chamber being 200 μl. A preliminary kinetic study showed that an equilibrium between the two sides of the Visking® dialysis membrane was achieved within 3 hr. No significant binding ($\leq$2%) to the dialysis membrane or the cells walls of the apparatus was observed. Isoxicam was used over a range of concentrations from 1 to 150 μM. ^{14}C isoxicam was directly diluted in phosphate buffer. At the end of each experiment, concentrations in each dialysis chamber were measured with a liquid scintillation counter (Packard Tricarb 460 CD). Isoxicam was dialysed either alone or in the presence of different concentrations of competitive binding inhibitors dissolved in buffer pH 7.4.

Fluorescence studies. Fluorescence measurements were made in an Aminco SPF 500 spectrofluorimeter. The fluorescence of solutions containing isoxicam (10 μM) and HSA (10 μM) was measured at room temperature before and after addition of drugs to concentrations of 10, 20, 30, 40 and 50 μM. The wavelength of excitation was 380 nm and the fluorescence was measured at 480 nm.

Determination of binding parameters. The equilibrium binding data were fitted to different mathematical models, namely the site-specific model and the stepwise equilibrium model.

The site-specific model. For m classes of independent binding sites (model of Scatchard), the bound concentration of drug (B) is related to the free concentration of drug (F) by the relationship:

$$\frac{B}{R} = \sum_{i=1}^{m} n_i \frac{K_i . F}{1 + K_i . F} \qquad (1)$$

where R is the concentration of total protein, and n_i and K_i are the number of binding sites and the association constant of the ith class of sites. However, to be theoretically valid, the Scatchard model should have integer values for n_i, implying the existence of several discrete binding sites. Each n_i was given a value of 1 then considering m binding sites, Eqn (1) may be transformed as:

$$\frac{B}{R} = \sum_{i=1}^{m} \frac{K_i . F}{1 + K_i . F}. \qquad (2)$$

The stoichiometric model. Another approach to the analysis of multiple binding is to consider each drug-protein complex (PD_i), including either 1, 2, or N bound drug molecules per molecule of protein, as different species of binder which interact with the drug according to a specific association constant, that is:

$$P + D \overset{K_1}{\rightleftharpoons} PD_1$$
$$PD_1 + D \overset{K_2}{\rightleftharpoons} PD_2 \qquad (3)$$
$$PD_{N-1} + D \overset{K_N}{\rightleftharpoons} PD_N$$

where N is the maximum number of binding reactions.

In this model, no hypothesis of discrete and independent binding sites is necessary, and the model is able to describe positive or negative cooperativity phenomena in the binding process. The bound and free drug concentrations are related as follows:

$$\frac{B}{R} = \frac{1 . K_1 . F^1 + 2 . K_1 . K_2 . F^2 + N . K_1 .. K_N . F^N}{1 + K_1 . F^1 + K_1 . K_2 . F^2 + K_1 .. K_N . F^N} \qquad (4)$$

Estimation of binding parameters. The binding parameters were estimated by iterative processes, Simplex or Gauss–Newton algorithms [12], that minimized the sum of squares of weighted deviations (s) of q observations, as follows:

$$s = \sum_{i=1}^{q} [(B_i(\text{obs}) - B_i(\text{calc}))/B_i(\text{obs})^{\omega}]^2 \qquad (5)$$

The weighting exponent, ω, was given a value between 0 and 1, the value 0 meaning that the error is independent of B, and 1 that the error is proportional to B. The value of ω was chosen in order to obtain a randomized distribution of the experimental points on the calculated curve. The value 0 was satisfactory. The estimated parameters were expressed as the mean ± SD of 3–5 determinations.

RESULTS

The binding parameters of isoxicam-HSA interaction

The different values obtained starting from the same experimental data (equilibrium dialysis experiments) are shown in Table 1. Using the integer coefficient model with fixed values for n_1 and n_2, we get two different association constants: the sum of which is close to the first stoichiometric binding constant. This comparison is possible when the free drug fraction is low compared to the protein concentration as demonstrated by Honoré and Brodersen [13].

Considering the site oriented evaluation without fixing n, the relevant data have the best accuracy,

Table 1. Binding parameters of isoxicam to HSA determined according to the different mathematical models (as described in materials and methods section)

Site specific model					
A: non-integer value					
n		K_a (M^{-1})			Sum of squares
1.92		58057			9.960
B: integer value					
n_1	n_2	K_{a1} (M^{-1})	K_{a2} (M^{-1})		Sum of squares
1	1	80363	33280		11.103
Stoichiometric model					
		K_1 (M^{-1})	K_2 (M^{-1})	K_3 (M^{-1})	Sum of squares
		114222	23840	0.03	11.103

Table 2. Affinity constants and apparent affinity constants of isoxicam (Isx) alone and in presence of warfarin 10 and 30 μM, diazepam 30 and 50 μM (A), arachidonic acid and palmitic acid at 5, 10 and 25 μM (B)

		Site I (warfarin)	Site II (diazepam)
(A)		K_{a1} (M^{-1})	K_{a2} (M^{-1})
^{14}C-Isoxicam (Isx)		51033	41380
Isx + warfarin 10 μM		28805	41380
Isx + warfarin 30 μM		11200	41380
Isx + diazepam 30 μM		51033	19990
Isx + diazepam 50 μM		51033	10200
(B)	FFA/HSA	K_{a1} (M^{-1})	K_{a2} (M^{-1})
^{14}C-Isoxicam (Isx)	0	57540	52420
Isx + arachidonic acid 5 μM	0.24	78750	32920
Isx + arachidonic acid 10 μM	0.48	110000	20470
Isx + arachidonic acid 25 μM	1.19	166000	6210
Isx + palmitic acid 5 μM	0.27	107070	43111
Isx + palmitic acid 10 μM	0.55	91988	16499
Isx + palmitic acid 25 μM	1.38	45123	6092

These parameters were determined simultaneously in a global estimation using the Scatchard model with integer values of n ($n_1 = n_2 = 1$).

i.e. the smallest sum of squares, and are also very close to the other values (when n is fixed). Thus the hypothesis of two binding sites for isoxicam seemed likely to be correct.

The isoxicam binding sites as evaluated by equilibrium dialysis experiments

The Table 2 indicates the respective values obtained using the site oriented model when fixing the number of binding sites. Figure 2 shows clearly that competitive binding antagonism occurs with both warfarin and diazepam vs isoxicam as the total number of sites remains constant and K_a decreases when the antagonist is added. Table 2 shows evidence of antagonism at both site I (antagonism by warfarin) and site II (antagonism by diazepam). Furthermore, the effects on the two sites seemed independent. Arachidonic acid increases K_{a1} and decreases K_{a2} showing that the first binding site is the warfarin one or a near locus, the second being the diazepam one. The same effect is obtained with palmitic acid 5 and 10 μM. However at higher concentrations, the enhancing effect on site I binding capacity decreases and stops (for palmitic acid 25 μM).

The fluorescence quenching of isoxicam–HSA interaction

A mixture of isoxicam (10 μM) and HSA (10 μM) excited at 380 nm emitted light at 480 nm, whereas isoxicam did not exhibit fluorescence in the free state in buffer at these wavelengths. Figure 3 shows the effects of binding site probes and palmitic acid on

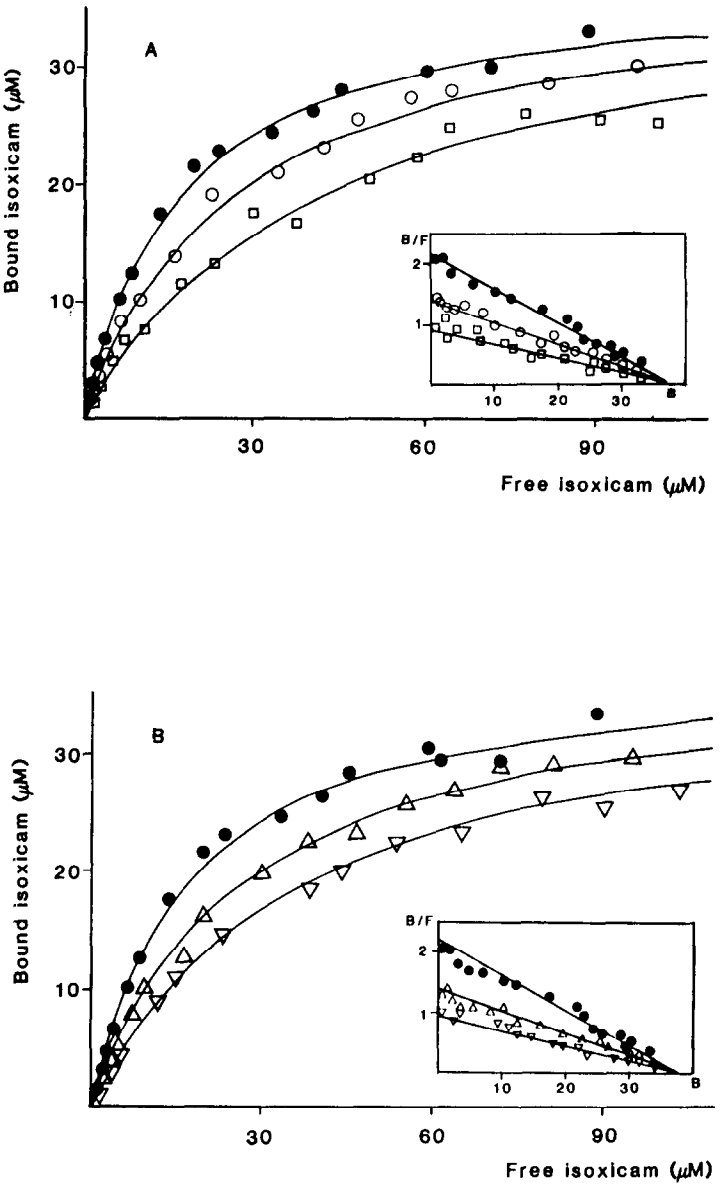

Fig. 2. Binding of isoxicam (1–150 μM) to HSA (20 μM) either alone (●) or with added diazepam (A) 30 μM (○) or 50 μM (□), or with added warfarin (B) 10 μM (△) or 30 μM (▽).

the fluorescence of isoxicam. Phenylbutazone, azapropazone and warfarin as well as diazepam and ibuprofen induce displacement of isoxicam. The fluorescence of isoxicam also decreases in presence of palmitic acid, the effect being small at ratios of palmitic acid to HSA of up to 3:1 with a large effect occurring at higher ratios.

These results support the above dialysis observations on the location of isoxicam binding sites.

DISCUSSION

The number of isoxicam binding sites on HSA

Considering the respective sums of squares of the three methods of analysis the best fit is found with the Scatchard derived method where n values are not previously fixed as integer numbers. Thus the most accurate determination is $n = 1.92$. However this value may be not statistically different from 2 or it may mean that one HSA site is not completely preformed before isoxicam binding as it was shown previously for various NSAIDs [13]. It may also be due to the flexibility of the albumin molecule which may accommodate its structure to the drug conformation. Furthermore, the data are adequately fitted because the free parameter model has enough degrees of freedom to conform to the variations of the experimental data.

The two other analyses gave a similar fit. It seems, however, that the stepwise equilibrium model (or

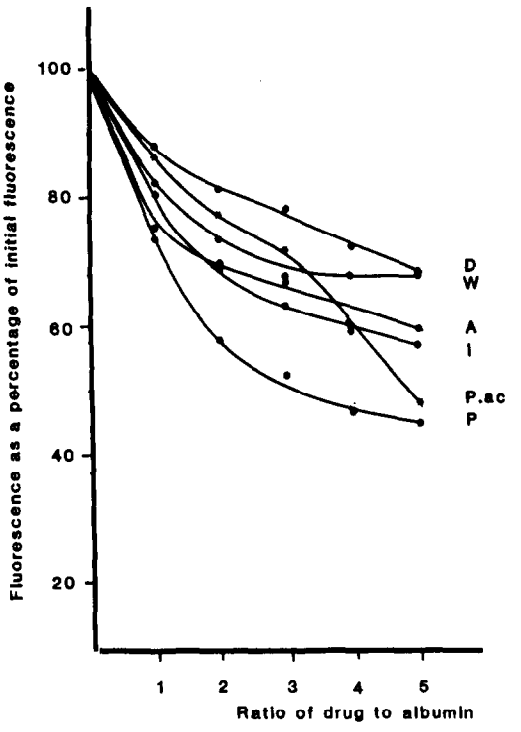

Fig. 3. Drug-induced changes in fluorescence of isoxicam bound to HSA. The fluorescence of solutions containing 10 μM HSA and 10 μM isoxicam in phosphate buffer pH 7.4, was measured before and after addition of drugs. The drugs used were: A, azapropazone; P, phenylbutazone; W, warfarin; D, diazepam; I, ibuprofen; P. ac, palmitic acid.

the stoichiometric one) is more accurate because it is more general: it does not imply preformed binding sites irrespective of cooperative or anticooperative mechanisms of binding [13, 14]. In the case of isoxicam, it describes three different drug complexes. The first one has a stoichiometric constant of 114216 M^{-1} which is similar to the sum of the association constants determined with the site specific model using integer values of n ($\sum_{i=1}^{2} n_i K_i$ = $(1 \times 80363) + (1 \times 33280) = 113643\ M^{-1}$). From the decreasing magnitude of the three stoichiometric constants (Table 1), we conclude that no positive cooperativity occurs during the isoxicam–HSA interaction and that the drug is bound to at least two sites.

The location of isoxicam binding sites on HSA

The respective values of K_{a1} and K_{a2} can be differentiated by appropriate analysis but are close enough to expect simultaneous binding to the two sites. K_{a_1} and K_{a_2} values are in a ratio of about 4, close enough for isoxicam to bind to the two sites at therapeutic plasma levels [9]. Both equilibrium dialysis and fluorescence data show that isoxicam binds to site I or near it and to site II of HSA. Considering site I, it seems that isoxicam binds probably to the azapropazone locus as better fits were obtained with this drug than with warfarin. Palmitic acid is classically used to differentiate binding to site I and II [5]. Binding of isoxicam was increased at one site and decreased at the other consistent with palmitic acid effects on other site I and II ligands [5]. The effects of palmitic acid on bound isoxicam fluorescence seem to be a combination of effects at the two binding sites. Interestingly, arachidonic acid caused similar effects to palmitic acid but they were more pronounced allowing the clear differentiation of the two binding sites. The fact that no apparent positive cooperativity occurred cannot exclude either a negative one or other indirect interactions including conformational changes or variations in electrostatic charges. This was recently reported for fatty acid-induced conformational HSA states, evidenced either by warfarin [15] or by oxyphenylbutazone [16] bindings.

In conclusion, the simultaneous binding of isoxicam to sites I (or near it) and II of HSA has been demonstrated. No apparent positive cooperativity exists between the two binding processes. The comparative use of two different calculation models, site oriented and stoichiometric, provides a useful tool to clarify the binding processes, and may be extended to other drugs which have multiple binding sites on HSA as determined by Scatchard analysis.

Acknowledgements—We wish to thank Warner Lambert Company, le Ministère de l'Education Nationale, le Ministère de la Santé for their grants. We gratefully acknowledge Prof. D. J. Birkett for his help both in the discussion of the results and in the translation of the manuscript.

REFERENCES

1. Sudlow G, Birkett DJ and Wade DN, The characterization of two specific drug binding sites on human serum albumin. *Mol Pharmacol* **11**: 824–832, 1975.
2. Sjöholm I, Ekman B, Kober A, Ljungstedt-Pahlman I, Seiving B and Sjödin T, 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.
3. Fehske KJ, Müller WE and Wollert U, The location of drug binding sites in human serum albumin. *Biochem Pharmacol* **30**: 687–692, 1981.
4. Sudlow G, Birkett DJ and Wade DN, Further characterization of specific drug binding sites on human serum albumin. *Mol Pharmacol* **12**: 1052–1061, 1976.
5. Wanwimolruk S, Birkett DJ and Brooks PM, Structural requirements for drug binding to site II on human serum albumin. *Mol Pharmacol* **24**: 458–463, 1983.
6. Wanwimolruk S and Birkett DJ, Structure binding relationships for site I and II on albumin in protein binding and drug transport. In: *Protein Binding and Drug Transport, Symposia Medica Hoechst No. 20, Arvor, Algarve, Portugal, 24–28 September 1985* (Eds. Tillement JP and Lindenlaub E), pp. 133–151. Schattauer, Stuttgart–New York, 1986.
7. Urien S, Albengres E and Tillement JP, Serum protein binding of valproic acid in healthy subjects and patients with liver disease. *Int J Clin Pharm Ther Toxicol* **19**: 319–325, 1981.
8. Albengres E, Urien S, Riant P, Marcel GA and Tillement JP, Binding of two anthranilic acid derivatives to human albumin, erythrocytes and lipoproteins: evidence for glafenic acid high affinity binding. *Mol Pharmacol* **31**: 294–300, 1987.
9. Downie WW, Comparative studies of isoxicam in rheumatoid arthritis. *Semin Arthritis Rheum* **13**: 148–153, 1983.

10. Reese RW, Long-term studies of isoxicam in the treatment of rheumatoid arthritis. *Am J Med* **79**: 12–16, 1985.
11. Posner SL, Six weeks, double-blind, placebo-controlled and long-term, open-label multicenter study of isoxicam in treatment of degenerative joint disease. *Am J Med* **79**: 17–23, 1985.
12. Zini R, D'Athis P, Barré J and Tillement JP, Binding of indomethacin to human serum albumin. Its non displacement by various agents, influence of free fatty acids and the unexpected effect of indomethacin on warfarin binding. *Biochem Pharmacol* **28**: 2661–2665, 1979.
13. Honoré B and Brodersen R, Albumin binding of anti-inflammatory drugs. Utility of a site oriented versus a stoichiometric analysis. *Mol Pharmacol* **25**: 137–150, 1984.
14. Fletcher JE and Spector AA, Alternative models for the analysis of drug-protein binding. *Mol Pharmacol* **13**: 387–399, 1977.
15. Chakrabarti SK, Cooperativity of warfarin binding with human serum albumin induced by free fatty acid anion. *Biochem Pharmacol* **27**: 739–743, 1978.
16. Dröge JHM, Janssen LHM and Witting J, The fatty-acid induced conformational states of human serum albumin investigated by means of multiple co-binding of protons and oleic acid. *Biochem J* **250**: 443–446, 1988.