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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

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Abstract—Binding of indomethacin to HSA was studied *in vitro* by equilibrium dialysis. Our results show that binding to HSA is 97 per cent at therapeutic levels. Binding is characterized by several saturable numbers of binding sites (n = 8) with a moderate association constant ($K = 1.2.\ 10^4\ M^{-1}$) and by another non-saturable phenomenon ($nK = 4200\ M^{-1}$). Furthermore, acetylsalicylic acid (ASA), salicylic acid (SA), 1 anilinonaphtalene-8-sulfonic acid (ANS), chlorophenoxyisobutyrate (CPIB), digitoxin, tryptophan and warfarin do not alter indomethacin binding. On the other hand, free fatty acids (FFA) decrease indomethacin binding in human serum albumin (HSA). Moreover indomethacin markedly decreases warfarin binding.

Indomethacin is one of the most potent anti-inflammatory drugs. It belongs to a group of nonsteroidal agents and, like these drugs, it is a weak acid which is highly bound (> 95%) to human serum albumin (HSA) [1]. Many authors [1–3] have determined the binding parameters for indomethacin including *n*, the number of binding sites and *k*, the association constant (or affinity). Since a comparison of these results shows many discrepancies, a major goal of this study was to re-examine this problem by measuring these parameters again.

In therapeutics, indomethacin is sometimes associated with other anti-inflammatory drugs and so it seems useful to know whether its albumin binding sites are shared with other substances that are weak acids. In this regard, conflicting results have been obtained with acetylsalicylic acid (ASA) [4]. A decrease in the peak plasma level of indomethacin in humans when ASA was co-administered had been reported. A displacement of the plasma bound form of indomethacin by the ASA can explain this variation. However, this was not found by other authors [5,6]. So, it became important to know whether these two acidic drugs are bound to the same HSA sites whether binding competition can occur between them. We also checked possible interactions with other drugs including non ionizable and basic substances.

MATERIALS AND METHODS

1. *Experimental methods*

a. *Indomethacin binding.* Indomethacin binding to HSA (Sigma A—1887) was studied by equilibrium dialysis. The experiments were carried out at 37°C, pH 7,4 (phosphate buffer, M/15) for 4 hr, under constant stirring at 20 rev/min. (Dianorm® apparatus). No significant binding was observed to the dialysis tubing (Visking®).

Indomethacin was used over a wide range of concentrations (1–3000 μM or 0.36–1075 μg/ml). Solutions were prepared by isotopic dilution of a constant amount of ^{14}C-labelled indomethacin (13,77 mCi/mmole, Merck, Sharp & Dohme) with increasing amounts of unlabelled drug. At the end of each experiment, concentrations in each compartment were measured by liquid scintillation counting (Packard Tricarb Liquid scintillation Spectrometer 3320). Two human serum albumin preparations containing different concentrations of free fatty acid (FFA) expressed as palmitic acid were used: HSA I (A-2386 Sigma) contained 3.45 moles FFA per mole of HSA while HSA II (A—1887 Sigma) contained 0.04 mole FFA per mole of HSA.

A human plasma pool was used with following characteristics: total proteins 68 g/l, serum albumin 40 g/l (580 μM) and small amounts of FFA, less than 0.04 mole per mole of albumin. FFA were measured by the method of Duncombe [7]. It was used for studying the effect of bilirubin on indomethacin binding. Bilirubin (Sigma) is solubilised in the plasma pool to a final concentration of 17 μM (10 μg/ml).

b. *Interaction between indomethacin and other drugs.* With HSA II (poor in FFA), several different experiments were carried out using indomethacin alone or in the presence of salicylic acid (SA) (Rhone-Poulenc), acetylsalicylic acid (ASA) (Rhone-Poulenc), chlorophenoxyisobutyric acid (CPIB) (Imperial Chemical Industries), digitoxin (Nativelle), warfarin (Merrell) or 1-anilinonaphthalene-8-sulfonic acid (ANS) (K.K. Laboratories). The range of indomethacin concentrations used was 0.28–280 μM (0.1–100 μg/ml) and all the studied substances were dissolved in HSA I and II (2 g/l or 29 μM) to a final concentration of 50 μM. The binding of ^{14}C-warfarin

(51 mCi/mmole, Amersham) over a range of 0.75–200 μM to HSA II at a concentration of 2 g/l was also studied. The same experiments were also repeated with 500 and 1000 μM of indomethacin.

2. Computation of binding parameters

Let the concentration of HSA be R and the total concentration of indomethacin be T. At equilibrium we measure the bound (B) and free (F) concentrations of this drug. Let us consider that HSA has two independent classes of binding sites simultaneously available for indomethacin. The binding of indomethacin to the first class of sites is saturable; the bound concentration (B_1) of this drug is related to its free concentration (F), as shown in the following equation:

$$B_1 = K_1 (n_1 R - B_1) F \quad (1)$$

where n_1 is the number of binding sites and K_1, the affinity constant of this drug for the first class of binding sites. The binding of indomethacin to the second class of sites is non saturable so that the bound concentration (B_2) of this drug is related to its free concentration (F) by the following equation:

$$B_2 = (K_2 n_2) RF \quad (2)$$

where the symbol ($K_2 n_2$) stands for the apparent affinity of a drug for the second class of binding sites, (both K_2 and n_2 symbolize one parameter). The total bound concentration (B) of indomethacin is the following:

$$B = B_1 + B_2. \quad (3)$$

Now, let us write:

$$\bar{B} = \frac{B}{R}. \quad (4)$$

Then if we consider both equations (1) and (2), we can write the following equation:

$$\bar{B} = \frac{K_1 n_1 F}{1 + K_1 F} + (K_2 n_2) F \quad (5)$$

This plot seems interesting because it allows the parameters estimation based on a linear transformation of measured values. This method has the advantage to weight the results mainly by the error of the measurement. On the contrary, the classical Scatchard plot ($\bar{B}/F$ vs $\bar{B}$) tends to disregard the points corresponding to the high free ligand concentrations: these high concentrations are measured with accuracy but give $\bar{B}/F$ values extremely low. The efficiency of this plot to estimate n_1, K_1 and (n_2 K_2) can be summarized as following. With a free ligand concentration (F) close to zero, equation (5) becomes approximately:

$$\bar{B} \simeq n_1 K_1 F. \quad (6)$$

So, the product $n_1 K_1$ represents the slope of the tangent from the origin. On the other hand, with a high free ligand concentration (F), we can write:

$$\bar{B} \simeq n_1 + (n_2 K_2) F. \quad (7)$$

So, n_1 and ($n_2 K_2$) can be estimated by drawing the tangent to the curve over the range of high free ligand concentrations. Consequently, the estimation of the parameters is valid provided that these tangents can be drawn accurately. Finally, a curve $\bar{B} = f(F)$ is plotted and then all parameters n_1, K_1 and ($K_2 n_2$) were estimated by means of the non linear least squares method using a Gauss-Newton algorithm.

RESULTS

1. Binding of indomethacin to HSA and plasma

a. *Determination of binding parameters.* Binding of indomethacin over the range of 1–3000 μM was studied at a HSA II concentration of 2 g/l (29 μM). The binding percentages decreased from 83 to 16 per cent when indomethacin concentration raised from 1 to 1000 μM. Figure 1 shows firstly, a saturation of indomethacin binding sites to HSA II and secondly, a linear increase of bound indomethacin concentration observed with the highest concentrations of drug. From these results, we can determine two classes of binding sites: the first one being saturable while the second one

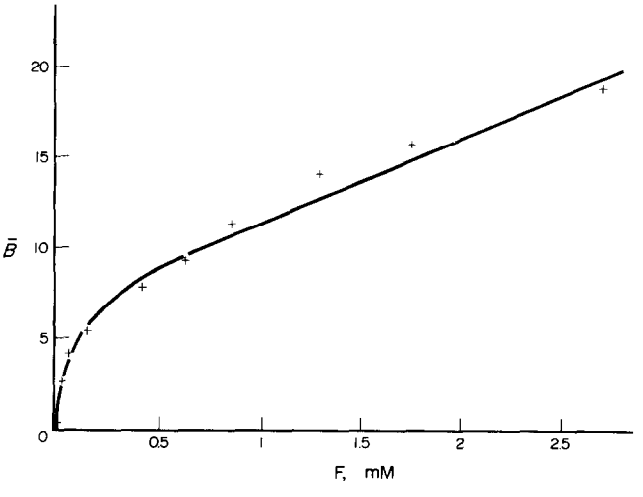

Fig. 1. Binding of indomethacin (1–3000 μM) to HSA II (29 μM). $\bar{B}$ represents the ratio: [Bound indomethacin]/[HSA] and F is free Indomethacin concentration. Each result is the mean of six determinations.

Table 1. Binding percentages of indomethacin

μM	Indomethacin concentrations or (μg/ml)	HSA I	HSA II	HSA II + Bilirubin	Plasma
0,7	(0.25)	95.2† ± 0.1	97.4 ± 0.1	97.2 ± 0.1	97.3 ± 0.1
2,8	(1)	95.0† ± 0.1	97.4 ± 0.1	97.2 ± 0.1	97.2 ± 0.1
140	(50)	92.9*† ± 0.1	96.7* ± 0.1	96.5* ± 0.1	96.7* ± 0.1

Binding percentages of indomethacin to HSA I (580 μM), HSA II (580 μM), HSA II with bilirubin at 17 μM and to plasma (HSA at 580 μM). Means (± S.D.) of five determinations are compared using Student's test.

*: significant decrease ($P < 0.001$) as compared with values of the same vertical column with 0.7 and/or 2.8 μM indomethacin concentrations and the same protein solutions.

†: significant decrease ($P < 0.001$) as compared with values obtained with the same indomethacin concentrations but with other protein solutions (same horizontal line).

is non-saturable. It appears that the first class of binding site has a rather high number of sites $n_1 = 7.8 \pm 0.7$ and a moderate affinity, $K_1 = 12775 \pm 3620\ M^{-1}$ while the second class of site has $n_2 K_2 = 4200 + 200\ M^{-1}$, 25 times smaller than that of the first class, although this class has a high binding capacity.

b. *Comparison of the binding of indomethacin to HSA II and plasma.* At therapeutic levels of indomethacin (0.25–1 μg/ml), we found that binding percentages to HSA II and to plasma (the HSA concentration in plasma being 40 g/l) were both 97.3%. So, there is no difference between binding to HSA II and to plasma. However, when we used a higher concentration of indomethacin (50 μg/ml or 140 μM), we noticed a significant decrease ($P < 0{,}001$) of binding percentage both with HSA II and plasma (Table 1).

2. *Interactions between indomethacin and other substances*

As shown in Figs. 2 and 3, the binding of indomethacin is not altered by acetylsalicylic acid (ASA), salicylic acid (SA), chlorophenoxyisobutyric acid (CPIB), digitoxin, tryptophan and 1-anilinonaphthalene-8-sulfonic acid (ANS). Binding percentages ranged from 88 to 45 per cent.

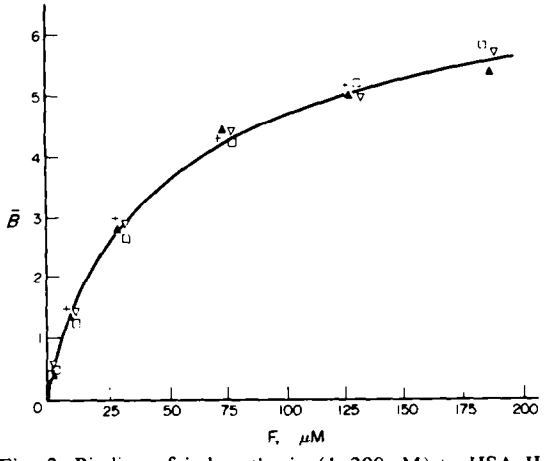

Fig. 2. Binding of indomethacin (1–200 μM) to HSA II (29 μM), alone (+——+) with ASA (▽— · ·▽), with S.A. (▲——▲) and with C.P.I.B. (□——□) at 50 μM. Each result is the mean of four determinations.

3. *Interaction between indomethacin and warfarin*

As can be seen from Fig. 4 high concentration of warfarin did not alter the binding of indomethacin: from 1 to 3000 μM, the binding percentage decreased from 83 to 16 per cent.

On the other hand, if we estimate the apparent parameters of indomethacin as n'_1, K'_1 and $(n_2 K_2)'$, when warfarin is added we obtained the same results that were observed without warfarin.

4. *Interaction between warfarin and indomethacin*

As shown in Fig. 5, the binding of warfarin is strongly inhibited by indomethacin. At the concentrations of warfarin employed, the binding percentage of this anticoagulant alone decreased from 89 to 34 per cent. In the presence of 500 μM of indomethacin, its binding percentage decreased from 30 to 16 per cent. So, at the lowest concentration of warfarin (0.75 μM) the addition of indomethacin (500 μM) produced a sevenfold increase in the concentration of free warfarin. Similarly, with 1000 μM of indomethacin, we obtained an eightfold increase of free warfarin. Thus, the binding of warfarin is obviously decreased by indomethacin.

5. *Interaction between indomethacin, FFA and bilirubin*

At the concentrations used, bilirubin did not displace bound indomethacin (Table 1). In contrast, high FFA concentrations did displace indomethacin in a non competitive fashion.

DISCUSSION

In order to model the binding of indomethacin, we used a new computing method including both saturable and non-saturable binding phenomena. This procedure revealed a rather high number of saturable binding sites ($n = 8$) on HSA. However, the affinity constant of indomethacin for those sites is relatively low compared to that of other anionic drugs [8] whose total binding percentages to HSA at 40 g/l are about the same. Although Mason *et al.* [1] used a narrower range (160–2000 μM) of indomethacin concentrations they also found two classes of binding sites. Their second class of sites have the same characteristics ($n = 7$, $K = 14000\ M^{-1}$) as those of our first class. However the fact that these authors also found a high affinity

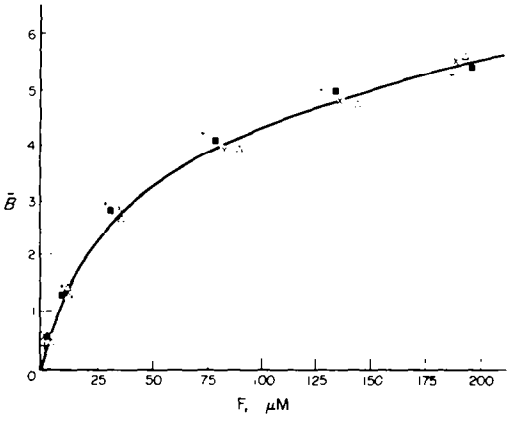

Fig. 3. Binding of indomethacin (1–200 μM) to HSA II (29 μM), alone (+ ——+) with A.N.S. (△– — △), with digitoxin (■——■), and with tryptophan (×– —×) at 50 μM. Each result is the mean of four determinations.

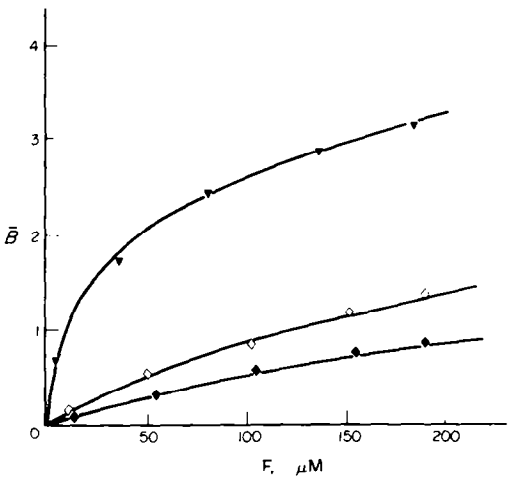

Fig. 5. Binding of warfarin (0.75–200 μM) to HSA II (29 μM), alone (▼– – ▼) and with indomethacin at 500 μM (◇——◇) or at 1000 μM (◆– – –◆).

class ($K = 3.10^5\ M^{-1}$) is probably due to different experimental conditions, i.e. HSA concentration of 23.5 g/l, use of ultra-filtration method and GLC measurement of indomethacin. Our results are in disagreement with those obtained by Hvidberg *et al.* [3], who found 15 sites with an affinity constant of 860 M^{-1}. They also used equilibrium dialysis method but with very high concentrations of both indomethacin (1–10 mg/ml) and HSA (40 g/l). At therapeutic plasma concentrations of indomethacin, from 1 to 2 μg/ml, it is impossible to saturate the first class of binding sites when HSA is in normal range, 40 g/l. Furthermore, any binding to the second binding sites class is undetectable. The binding percentage at these therapeutic concentrations remains constant at 98 per cent. These results are in accordance to the finding of Hultmark *et al.* [2]. Non pharmacological indomethacin concentrations, 50 μg/ml for instance, must be used to detect a slight but significant binding percentage decrease, 96.5 per cent (Table 1). This observation can be explained by a binding quantitatively more important on the second class than on the first class of binding sites.

To our knowledge, this is the first report of a second non saturable class of binding sites on HSA for an acidic drug, which also exhibits higher affinity saturable binding.

Some authors [1, 2, 9] have reported that competition occurs between indomethacin and salicylates, tryptophan and tryptophan congeners. These conclusions suggest that these drugs share at least one class of binding sites and that, at their level, for the same molar concentrations, their affinity constants were higher than those of indomethacin. In this respect, our experiments were run under conditions where displacement of bound indomethacin must be seen. The results obtained showed that this event is unlikely to occur. Furthermore, we checked the possibility for indomethacin to share at least one of the two specific sites of acidic drugs as described by Sudlow *et al.* [10]. Using ANS and CPIB as respective probes of these sites [11], it was impossible under our experimental conditions, to observe any displacement of bound indomethacin. This result suggests that indomethacin binding sites are different from those of acidic drugs. Bilirubin, which is

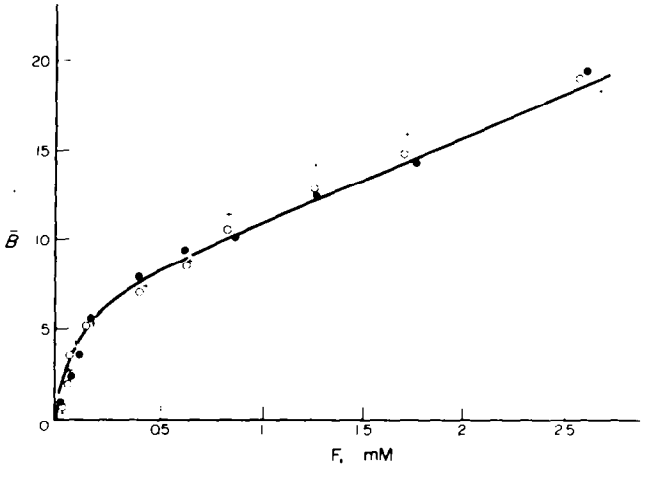

Fig. 4. Binding of indomethacin (1–3000 μM) to HSA II (29 μM), alone (+ — +) and with warfarin at 200 μM (○– –○) or at 1000 μM (●——●). Each result is the mean of four determinations.

known to have one high affinity binding site, $K = 10^8\ M^{-1}$ [12] shared by acidic drugs, is also unable in the range of physiological concentrations, to displace bound indomethacin. Moreover, ASA which is known to induce a structural change of HSA [13–15] does not alter the indomethacin binding. Similarly, digitoxin, a non ionizable drug which is bound to HSA in a non saturable process*, is also unable to modify indomethacin binding.

The only effective agents which decreased indomethacin binding, were FFA. However their role is not quite clear. A number of previous reports have suggested that FFA binding sites are common with those of CPIB, warfarin and phenylbutazone [16–19] which are all acidic drugs. But another author [20], found that linoleic, palmitic and oleic acids have two different effects on warfarin binding to HSA. At low molar concentrations (FFA/HSA = 3) they increase its association constant, while at high concentrations FFA/HSA > 6, they decrease it in a competitive way. So he suggests for these FFA, two different classes of binding sites, the first being responsible for specific binding while the second is non specific shared with anionic drugs included warfarin. The binding of FFA to the first class seems to induce a structural change of HSA which results in an increased association constant of warfarin, by a positive cooperative effect.

Other reports also mention structural change of HSA induced by FFA [21–23]. In plasma FFA may act as stabilizers of HSA conformation. Our results seem to be consistent with this hypothesis. We also showed that warfarin can be displaced by indomethacin (Fig. 5).

Since warfarin does not displace indomethacin, this phenomenon cannot be classified as a competitive inhibition. This seems normal as warfarin has only one high affinity site, $K = 2.5.10^5\ M^{-1}$ [8] while indomethacin has 8 low affinity sites, $K = 10^4\ M^{-1}$. The displacement of warfarin by indomethacin can be explained by one of the two following hypothesis: Either a non competitive inhibition without any conformational change of HSA, or a conformational change of HSA induced by indomethacin. These two assumptions could be consistent with the following one. Indomethacin induced a modification of the warfarin binding sites structure; however, according to a non competitive inhibition model, the affinity constant of warfarin must be unchanged and simultaneously the number of binding sites must decrease. We observed that the warfarin affinity decreased from $K = 2.5 \cdot 10^5\ M^{-1}$ (warfarin alone) to $K = 3.10^3\ M^{-1}$ (warfarin with indomethacin 1000 μM) and that the number of binding sites do not vary significantly ($n = 1.3 \pm 0.2$ and 2.3 ± 0.3). Therefore indomethacin cannot be considered as a non competitive inhibitor, but as an inducer of structural change of albumin. However these results do not agree with those of Sjoholm *et al.*[24].

In conclusion, indomethacin is an acidic drug that is practically totally ionized at plasma pH and is highly bound to HSA. However its binding parameters are very different from those of other anionic drugs. The number of binding sites is relatively important while the affinity is relatively weak. Furthermore, indomethacin does not compete with other anionic drugs, but can prevent the binding of warfarin to HSA probably via an allosteric mechanism.

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