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BINDING OF CLOMETACIN TO HUMAN SERUM ALBUMIN

INTERACTIONS WITH CLOFIBRATE, INDOMETHACIN, SALICYLIC ACID AND WARFARIN

R. ZINI, J. BARRE, P. D'ATHIS* and J. P. TILLEMENT

Département de Pharmacologie, Faculté de Médecine de Paris XII, 8 rue du Général Sarrail, F-94010 Creteil Cédex, France; and *Département d'Informatique Médicale (Pr. Ag. L. Dusserre) Hôpital du Bocage, F-21034 Dijon Cédex, France

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Abstract—The binding of clometacin to human serum albumin (HSA) was studied *in vitro* by equilibrium dialysis. Our results show that binding to HSA is 99% at therapeutic levels. Binding is characterized by several numbers of binding sites ($n = 8$) with a moderate association constant ($K = 2.7 \times 10^4\ M^{-1}$) and by another non-saturable phenomenon ($nK = 4200\ M^{-1}$). Moreover, interactions were studied with many drugs. Clometacin binding was altered by indomethacin, warfarin, chlorophenoxyisobutyrate (CPIB) and salicylic acid (SA). Conversely, clometacin inhibited the binding of these drugs. Finally, all these results were compared with those previously obtained with indomethacin, a *positional* isomer of clometacin.

Clometacin is a positional isomer of indomethacin. Both drugs have the same pharmacological properties but the former seems to exhibit more analgesic than anti-inflammatory effects. They are extensively bound to human serum albumin (HSA). It was previously shown that binding of indomethacin to HSA despite its high ionization at physiological pH is different from that of other anti-inflammatory drugs [1]. Therefore, we thought it would be interesting to investigate also the HSA binding of its isomer, firstly to check HSA binding of clometacin and the relevant drug interactions, and secondly to compare binding characteristics of the two drugs in order to evaluate the effect of isomerization at this position on HSA binding (Fig. 1).

MATERIALS AND METHODS

1. *Experimental methods*

(a) *Clometacin binding*. Clometacin binding to HSA (Sigma A—1887) was studied by equilibrium dialysis. The experiments were carried out at 37°, 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®). HSA samples contained 0.04 mole of free fatty acid (FFA) expressed as palmitic acid per mole of HSA. Clometacin was used over a wide range of concentrations (0.7–2800 μM). Its solutions were prepared by isotopic dilution of a constant amount of [^{14}C]clometacin (5.15 mCi/mmole, Roussel-Uclaf, 98% pure checked by thin-layer chromatography: *n*-butanol–ethanol–ammonia buffer*, 40:10:20, v/v) 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).

A human serum pool was used with following characteristics: total proteins 70 g/l, serum albumin (600 μM), FFA (790 μM). FFA levels were determined by a gas chromatographic method. A Girdel® 3000 gas chromatograph equipped with a flame ionization detector and a Servotrace (Séfram®) recorder was used. Chromatography was carried out using a glass u-column 1.8 m long and 2 mm i.d. packed with 10% SP-216-PS on 100/120 mesh supelcoport [2]. Measurements were made with programmed temperature 170°–200° (5°/min). An internal standard of pentadecanoic acid was added to HSA and serum solutions. The extraction solvent used was chloroform–*n*-heptane–methanol (3:2:0.1, v/v). The organic phase was removed and evaporated to dryness. The residue was dissolved in 50 μl *n*-heptane, and 5 μl was injected into the gas chromatograph.

(b) *Interaction between clometacin and other substances*. With 600 μM HSA, five concentrations of clometacin (2.8, 14, 28, 56, 140 μM) were dialysed alone or in the presence of a final concentration of 1000 μM of palmitic acid (Sigma P—2010). It was solubilized in ethanol, then evaporated to dryness with nitrogen flux; the HSA solution was then added and gently stirred. FFA concentration was controlled by gas chromatography. With the serum pool, several different experiments were carried out using three concentrations of clometacin (2.8, 7 and 14 μM), alone or in the presence of a final concentration of 600 μM of chlorophenoxyisobutyric acid (CPIB) (Imperial Chemical Industries), 3.5 μM of diazepam (Ciba Geigy), 0.052 μM of digitoxin (Nativelle), 1.5 μM of furosemide (Hoechst), 15 μM of indomethacin (Merck Sharp & Dohme), 2200 μM of sal-

* Ammonia buffer was obtained by mixing equal volumes of 1.5 N NH_4OH and 1.5 N $(NH_4)_2CO_3$.

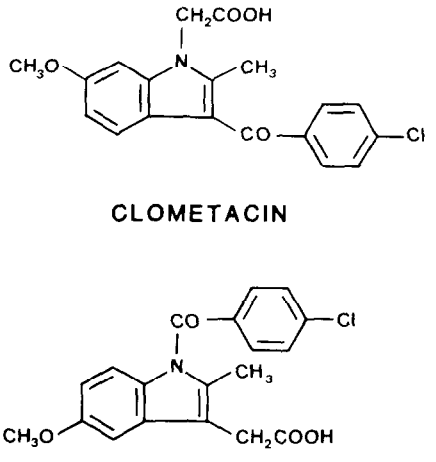

Fig. 1. Structural formulae of indomethacin and clometacin.

icylic acid (SA) (Rhone Poulenc), 10 μM of warfarin (Merrell), 600 μM of tryptophan (Sigma T0629) and 60 μM of bilirubin (Sigma B 4126). All the studied substances were dissolved in the serum pool.

More investigations of the interactions were carried out at 29 μM HSA. The binding of [^{14}C]clometacin was repeated in the presence of indomethacin (50, 100, 200 μM), CPIB (500, 1000, 2000 μM), warfarin (250, 500, 1000 μM) and SA (500, 1000, 2000, 3000 μM).

Conversely, the binding of all these inhibitors was studied as follows: [^{14}C]warfarin (51 mCi/mmole, Amersham Centre, 98% pure checked by thin-layer chromatography: toluene–methanol, 90:10, v/v) over a range of 0.8–400 μM with 50 and 100 μM of clometacin, [^{14}C]CPIB (22 mCi/mmole, CEA, 97% pure checked by thin-layer chromatography: chloroform–acetic acid, 95:5, v/v) over a range of 1.1–2325 μM with 50 and 100 μM of clometacin, [^{14}C]indomethacin (13.77 mCi/mmole, Merck Sharp & Dohme, 97% pure checked by thin-layer chromatography: *n*-butanol–ethanol–ammonia buffer, 40:10:20, v/v) over a range of 0.7–2800 μM with 100 and 200 μM of clometacin, [^{14}C]SA (25 mCi/mmole, CEA, 98% pure checked by thin-layer chromatography: *n*-butanol–acetic acid–water, 50:25:25, v/v) over a range of 1.7–3570 μM, with 50 and 150 μM of clometacin.

2. *Computation of binding parameters*

All binding parameters were estimated by means of the non-linear least-squares method using a Gauss–Newton algorithm [1]. The calculations were performed on a Tektronix 4051.

RESULTS

1. *Binding of clometacin to HSA and serum*

(a) *Determination of binding parameters.* Binding of clometacin over the range 0.7–2800 μM was studied at a HSA concentration of 29 μM. The binding percentages decreased from 93 to 18%. Figure 2 shows, on the one hand, a biphasic curve with first a saturation of the clometacin main binding sites and secondly, a linear increase of bound clometacin concentrations observed with the highest concentration of drug, and on the other hand, a Scatchard plot with two clear-cut classes of binding sites. The relevant binding parameters were $n_1 = 7.7 \pm 0.2$, $K_1 = 27{,}300 \pm 2100\ M^{-1}$ for the first saturable class, and $n_2K_2 = 4100 \pm 150\ M^{-1}$ for the second non-saturable class.

(b) *Comparison of the binding of clometacin to serum and to HSA with and without FFA.* The binding percentages of clometacin were measured at different concentrations in serum (the HSA concentration being 600 μM) and in two 600 μM HSA preparations—the first one practically free of FFA while the second one contained 1000 μM FFA. In

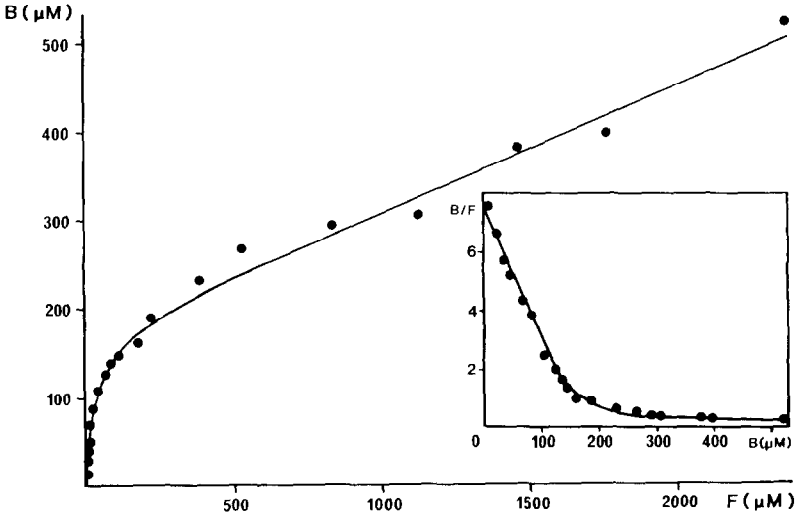

Fig. 2. Binding of indomethacin (0.7–2800 μM) to HSA (29 μM). B denotes the bound clometacin concentrations and F the free clometacin concentrations. Each result is the mean of five determinations. Insert: Scatchard plot shows two classes of binding sites.

Table 1. Binding percentages of clometacin to serum (HSA at 600 μM), HSA (600 μM) and HSA (600 μM) with palmitic acid (1000 μM)

Clometacin (μM)	Serum	HSA 600 μM	HSA 600 μM, palmitic acid 1000 μM
2.8	99.4 ± 0.1	99.5 ± 0.1	99.4 ± 0.1
14	99.5 ± 0.1	99.5 ± 0.1	99.5 ± 0.1
28	99.3 ± 0.1	99.4 ± 0.1	99.4 ± 0.1
56	99.4 ± 0.1	99.4 ± 0.1	99.4 ± 0.1
140	99.3 ± 0.1	99.3 ± 0.1	99.4 ± 0.1

Means (±S.D.) of five determinations.

the three experiments, the clometacin binding percentages remained constant, 94% (Table 1). First, it appears that HSA alone accounts for clometacin binding in serum, since the binding percentage in serum is equivalent to that of HSA. Secondly, FFA showed no influence on clometacin binding in the range of concentrations used.

2. *Interactions between clometacin and other substances*

(a) *Interactions in serum.* The binding of clometacin at therapeutic levels (2.8, 7, 14 μM) was studied in serum in the presence of drugs and endogenous compounds at usual therapeutic or physiological concentrations: warfarin (10 μM), furosemide (1.5 μM), diazepam (3.5 μM), digitoxin (0.052 μM), indomethacin (15 μM), SA (2200 μM), CPIB (600 μM), tryptophan (600 μM) and bilirubin (60 μM).

As shown in Table 2, the binding of clometacin was significantly inhibited by SA and CPIB ($P < 0.001$ and $P < 0.01$, respectively). The other compounds showed no influence on clometacin binding.

(b) *HSA interactions.* At 29 μM HSA, the binding of [^{14}C]clometacin over a wide range of concentrations (0.7–2800 μM) was altered by indomethacin (50, 100 and 200 μM), warfarin (250, 500 and 1000 μM), CPIB (500, 1000 and 2000 μM) and SA (500, 1000 and 2000 μM). As shown in Table 3, the number of binding sites of clometacin remained constant while its apparent affinity constant decreased as the molar concentrations of the inhibitors increased. These interactions can be classified as competitive inhibitions. Figure 3 illustrates these competitions.

Conversely, the binding of each labelled inhibitor was studied over a wide range of concentrations in the presence of several concentrations of clometacin at 29 μM HSA. As shown in Table 4, clometacin inhibited the binding of indomethacin, warfarin, CPIB and SA following competitive processes. As the clometacin concentrations increased, the number of sites of these drugs remained constant while their apparent affinity constants markedly decreased.

DISCUSSION

Indomethacin and clometacin have an identical number of HSA binding sites ($n = 8$). One would expect this finding since these two compounds are positional isomers (Fig. 1). But it is noteworthy that usually the number of these saturable binding sites belonging to the first class is much lower ($n = 1$ or 2) for most acidic drugs [3–6]. The affinity constant of this first class of binding sites of clometacin is

Table 2. Binding percentages of clometacin alone, and in the presence of warfarin (10 μM), furosemide (1.5 μM), diazepam (3.5 μM), digitoxin (0.052 μM), indomethacin (15 μM), SA (2200 μM), CPIB (600 μM), tryptophan (600 μM) and bilirubin (60 μM) to serum (HSA at 600 μM)

	Clometacin concentration (μM)		
	2.8	7	14
Clometacin alone	99.4 ± 0.1	99.4 ± 0.1	99.5 ± 0.1
Clometacin + warfarin (10 μM)	99.4 ± 0.1	99.3 ± 0.1	99.4 ± 0.1
Clometacin + furosemide (1.5 μM)	99.3 ± 0.1	99.4 ± 0.1	99.4 ± 0.1
Clometacin + diazepam (3.5 μM)	99.3 ± 0.1	99.4 ± 0.1	99.4 ± 0.1
Clometacin + digitoxin (0.052 μM)	99.5 ± 0.1	99.4 ± 0.1	99.3 ± 0.1
Clometacin + indomethacin (15 μM)	99.4 ± 0.1	99.4 ± 0.1	99.5 ± 0.1
Clometacin + SA (2200 μM)	96.8 ± 0.1*	96.7 ± 0.2*	96.7 ± 0.3*
Clometacin + CPIB (600 μM)	98.7 ± 0.1†	98.6 ± 0.1†	98.5 ± 0.1†
Clometacin + tryptophan (600 μM)	99.4 ± 0.1	99.3 ± 0.1	99.4 ± 0.1
Clometacin + bilirubin (60 μM)	99.4 ± 0.1	99.4 ± 0.1	99.4 ± 0.1

Means (±S.D.) of five determinations are compared using Mann and Whitney's non-parametric test.

* $P < 0.001$.

† $P < 0.01$.

Table 3. Apparent affinity constant of the saturable binding sites of clometacin in the presence of indomethacin (50, 100 and 200 μM), warfarin (250, 500 and 1000 μM), CPIB (500, 1000 and 2000 μM) and SA (500, 1000 and 2000 μM)

	K_1 (M^{-1})
Clometacin alone	27,300 ± 2100
Clometacin + indomethacin (50 μM)	13,000 ± 1500
Clometacin + indomethacin (100 μM)	8900 ± 940
Clometacin + indomethacin (200 μM)	5600 ± 570
Clometacin + warfarin (250 μM)	23,500 ± 3700
Clometacin + warfarin (500 μM)	11,500 ± 600
Clometacin + warfarin (1000 μM)	7300 ± 970
Clometacin + CPIB (500 μM)	9000 ± 1040
Clometacin + CPIB (1000 μM)	5480 ± 180
Clometacin + CPIB (2000 μM)	3540 ± 170
Clometacin + SA (500 μM)	11,520 ± 1680
Clometacin + SA (1000 μM)	9320 ± 1320
Clometacin + SA (2000 μM)	5260 ± 700

The affinity constant of clometacin alone is 27,300 ± 2100 M^{-1}. The number of saturable binding sites remains constant ($n = 8$) whatever the concentration of the inhibiting drug. Those parameters were determined with a wide range of concentrations (0.7–2800 μM). Each result is the mean of three determinations.

twice that of indomethacin, while nK values of the second class are similar for the two drugs (≈4100 M^{-1}). The latter result is not surprising since nK reflects more a partition coefficient between HSA and the drugs than a specific binding on clear-cut sites [7]. In addition to these observations, our results showing competitive inhibition between clometacin and indomethacin strongly suggest that these two drugs share the same binding sites.

The identical binding percentages measured in serum and in HSA solution show that HSA alone accounts for clometacin binding, as for indomethacin [1]. On the other hand, this result shows that the presence of FFA (790 μM) in serum does not influence clometacin binding. Moreover, the clometacin binding percentages to HSA with and without 1000 μM palmitic acid confirm this finding. Those results differ from those obtained with indomethacin, whose HSA binding was decreased in the presence of 2100 μM FFA [1]. In the present study, the discrepancy can be explained by lower FFA levels and a 2-fold affinity constant which leads to an increase of 2% in clometacin binding (99.5%) compared to that of indomethacin (97.4%) at the same concentration of 2.8 μM. Bilirubin and tryptophan, which are known to have one high affinity binding site shared by acidic drugs [8, 9], are also unable to displace bound clometacin in the range of physiological concentrations. Similarly, other drugs such as warfarin, furosemide, diazepam, digitoxin and indomethacin did not show any influence on clometacin binding at therapeutic levels. Although they were used at maximum therapeutic levels, their molar concentrations were too low to induce displacement. The total required concentration should be close to 500 μM, that is the concentration which saturates the first class of binding sites. This condition was fulfilled by CPIB (600 μM) and SA (2200 μM), at therapeutic levels. Clometacin binding was inhibited by these two drugs. More detailed investigations about interactions showed that clometacin binding was altered by indomethacin, warfarin, CPIB and SA at 29 μM HSA. These interactions can be classified as competitive inhibitors since the number of binding sites of clometacin remained constant and K decreased when the concentrations of the inhibitors increased. This classification is substantiated by the reverse experiments. The affinity constant of indomethacin, CPIB, warfarin and SA markedly decreased with increasing concentrations of clometacin while their number of binding sites remained identical.

As a very high affinity HSA binding site (≈2 × 10^6 M^{-1}) was observed by other authors [10] for indomethacin, our results with clometacin do not rule out the presence of such a site since the specific activity of the sample used was too low to enable us to detect it. It can be assumed that this site, if any, is likely to be common with the high affinity site of warfarin, CPIB and SA binding. Thus, it is clear that the concentrations of clometacin used (50 and 100 μM) might be sufficient for an important inhibition of warfarin, CPIB and SA binding, in view

Table 4. Affinity constants and apparent affinity constants of indomethacin (0.7–2800 μM), warfarin (0.8–400 μM), CPIB (1.1–2325 μM) and SA (1.76–3570 μM) alone and in the presence of clometacin at 50, 100 and 200 μM

	n_1	K_1 (M^{-1})
Indomethacin	7.8 ± 0.7	15,600 ± 3300
Indomethacin + clometacin (100 μM)	7.8 ± 0.7	12,800 ± 1970
Indomethacin + clometacin (200 μM)	7.8 ± 0.7	9700 ± 1450
Warfarin	1 ± 0.2	237,100 ± 77,400
Warfarin + clometacin (50 μM)	1 ± 0.2	104,910 ± 30,100
Warfarin + clometacin (100 μM)	1 ± 0.2	54,000 ± 15,200
CPIB	1 ± 0.2	274,140 ± 31,000
CPIB + clometacin (50 μM)	1 ± 0.2	125,630 ± 10,500
CPIB + clometacin (100 μM)	1 ± 0.2	84,050 ± 6100
SA	1.3 ± 0.2	45,670 ± 3500
SA + clometacin (50 μM)	1.3 ± 0.2	20,400 ± 2500
SA + clometacin (100 μM)	1.3 ± 0.2	11,250 ± 1700

Each curve is the mean of three determinations.

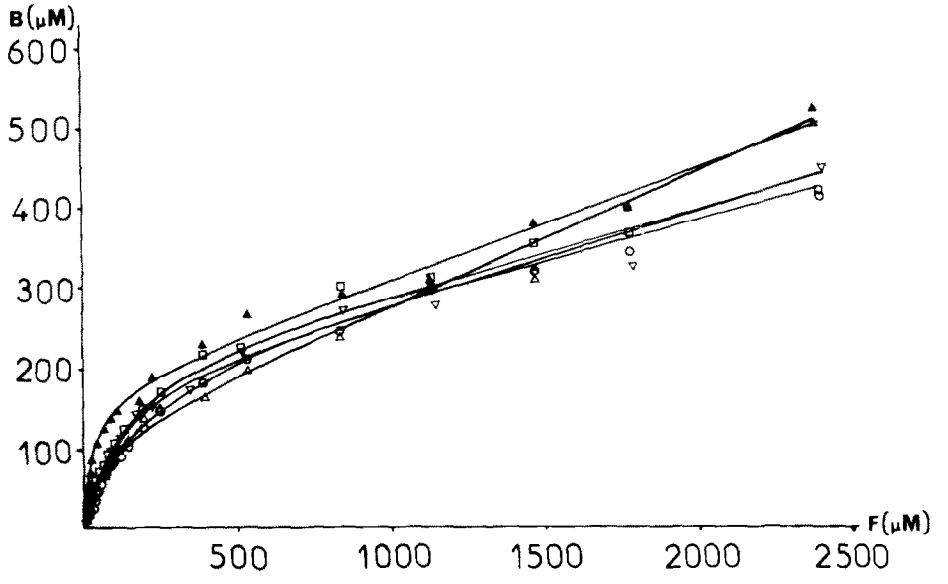

Fig. 3. Binding of clometacin (0.7–2800 μM) to HSA (29 μM) alone (▲—▲), with CPIB at 500 μM (□—□), with indomethacin at 200 μM (○—○), with SA at 2000 μM (△—△) and with warfarin at 500 μM (▽—▽).

of the higher affinity of the presumed site ($\simeq 2 \times 10^6\ M^{-1}$) for clometacin in comparison to that of warfarin ($\simeq 2.3 \times 10^5\ M^{-1}$), CPIB($\simeq 2.7 \times 10^5\ M^{-1}$) and SA ($4.5 \times 10^4\ M^{-1}$).

On the other hand, high concentrations of warfarin, CPIB and SA (500 and 1000 μM) would be necessary to displace it from its presumed high affinity site. Further, these assumptions are supported by the observation of the ability of indomethacin to displace clometacin at much lower concentrations (50 and 100 μM).

It is also possible that the saturable class of eight binding sites of clometacin completely or partially overlaps the high affinity site commonly shared by CPIB, SA and warfarin. In this situation, clometacin could be regarded as a competitive inhibitor of these drugs.

It could also be assumed that the eight saturable sites of clometacin are very close to the high affinity site of CPIB, SA and warfarin. Thus, the occupation of one or a part of those sites could lead to a conformational change of the high affinity site. In this situation, clometacin should be regarded as a non-competitive inhibitor. But this assumption does not hold true since our results show that the number of warfarin, CPIB or SA binding sites is constant while the affinity constant of these drugs decreases in the presence of clometacin. The same arguments can be put forward to account for the two sorts of inhibition between clometacin and indomethacin.

Finally, from our results clometacin binding (Table 3) was inhibited by warfarin at 250 μM. This is contradictory with the non-displacement of indomethacin used in the same range as clometacin (1–3000 μM) in the presence of warfarin at 200 and 1000 μM. Assuming that these two drugs share the same binding sites, these contradictory results have no explanation at present.

The results of the present study seem to show that CPIB, SA, warfarin, indomethacin and clometacin are bound to the same area of HSA. This is in accordance with the finding of Sjoholm *et al.* [11], which indicates that indomethacin, warfarin and SA share common binding sites.

There is evidence for the existence of at least two relatively specific sites on HSA to which anionic drugs bind [11, 12]. These two specific binding sites were identified by using two different techniques, probe fluorescence displacement [13] and HSA immobilization in microparticles [11]. Site I was shown to be specific for warfarin and site II for anti-inflammatory drugs. Indomethacin was found to be bound to both sites I and II [11]. So, the behaviour of clometacin is likely to be the same.

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