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PLASMA PROTEIN BINDING OF THE REVERSIBLE TYPE A MAO INHIBITOR CIMOXATONE (MD 780515)

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Abstract—Binding of a new selective reversible type A MAO inhibitor cimoxatone (MD 780515) to plasma proteins was studied *in vitro* by equilibrium dialysis. Binding to 580 μM human serum albumin (HSA) and to total plasma proteins was 93–96% and independent of cimoxatone concentration (0.15–207 μM). The drug was mainly bound to HSA with two binding sites and a moderate association constant ($K = 2.9 \times 10^4\ M^{-1}$). Free fatty acids did not modify cimoxatone binding to HSA. Cimoxatone was also moderately bound to isolated lipoprotein fractions; α_1-acid glycoprotein and γ-globulins did not play an important role in the binding of cimoxatone. MD 770222, the *O*-demethyl metabolite, appeared to be bound to HSA at the same binding sites as cimoxatone. However, no interaction occurred between the two compounds for 580 μM HSA. L-Tryptophan, bilirubin, the benzodiazepines flunitrazepam and oxazepam, imipramine and aspirin, did not displace cimoxatone from its binding sites. On the other hand, warfarin and phenylbutazone decreased cimoxatone binding to 29 μM HSA but no interaction occurred with 580 μM HSA.

Cimoxatone (MD 780515) (Fig. 1), (3-[4-(3-cyanophenylmethoxy) - phenyl] - 5(methoxymethyl) - 2-oxazolidinone), is a new selective and reversible inhibitor of type A monoamine oxidase (MAO) both in rat [1, 2] and man [3]. Cimoxatone is extensively metabolized in man [4]. Its plasma metabolite MD 770222 (Fig. 1), the *O*-demethyl derivative (3-[4-(3-cyanophenylmethoxy) - phenyl] - 5(hydroxymethyl)-2-oxazolidinone), is also a selective and reversible type A MAO inhibitor, but is seven to eight times less inhibitory than the parent compound [5]. A pharmacokinetic study has shown that after oral administration of 80 mg [^{14}C]cimoxatone to four healthy adult volunteers, peak plasma concentrations of the drug ranged from 0.8 to 1 mg/l. at 2–7 hr and those of the metabolites varied from 0.35 to 0.54 mg/l. at 24–30 hr. The plasma radioactivity was mainly due to cimoxatone and MD 770222 [6]. Since 96–85% of the total radioactivity was bound to plasma proteins (mostly to albumin) between 0.5 and 96 hr after dosing [7], the binding of both compounds was further investigated. The purpose of this study was to determine the binding parameters of cimoxatone for human serum albumin (HSA); binding to other plasma proteins (α_1-acid glycoprotein, lipoproteins and γ-globulins) was also investigated. The interaction between cimoxatone and its metabolite MD 770222 for albumin binding was examined, as well as the possible interactions with endogenous compounds (free fatty acids, bilirubin and L-tryptophan). Finally, a possible albumin-binding displacement of cimoxatone by several drugs (imipramine, aspirin, oxazepam, flunitrazepam, warfarin and phenylbutazone) was also studied.

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MATERIALS AND METHODS

1. *Cimoxatone binding.* Cimoxatone binding to plasma proteins was studied by equilibrium dialysis. Dialyses were carried out at 37° against an isotonic buffer solution of 0.05 M phosphate and 0.07 M NaCl, pH 7.4, for 2 hr in 1 ml Teflon cells (1 S) under constant stirring at 20 rev/min (Dianorm®, Diachema, Switzerland).

Solutions of cimoxatone were prepared by isotopic dilution of a constant amount of ^{14}C-labelled cimoxatone (11 mCi/mmole, C.E.A., Saclay, France) with increasing amounts of unlabelled drug. The drug was first dissolved in dimethyl sulfoxide, 3% of the aqueous solution, before addition of the buffer solution. Solubilisation was achieved by heating the solution at 40°. Under these conditions, the maximum concentration of cimoxatone in solution did not exceed 70 mg/l. At the end of each experiment, the [^{14}C]cimoxatone concentration in each compartment was measured by liquid scintillation counting with Unisolve (Packard Tricarb 3320 and Intertechnique SL 3000 spectrometers).

Preliminary experiments had demonstrated that equilibrium between plasma and buffer compartments was reached within 2 hr and that no degradation of cimoxatone occurred during dialysis. No significant binding to the cellulose dialysis membranes (Spectrapor 2®, Spectrum Med. Inc., U.S.A.) was observed.

All concentrations quoted refer to the initial concentration of ligand in buffer.

(a) *Binding to HSA*: Two HSA preparations containing different concentrations of free fatty acid (FFA) expressed as palmitic acid were used: HSA I (A-2386 Sigma) contained 3.45 mole FFA/mole of HSA; HSA II (A-1887 Sigma) contained 0.04 mole

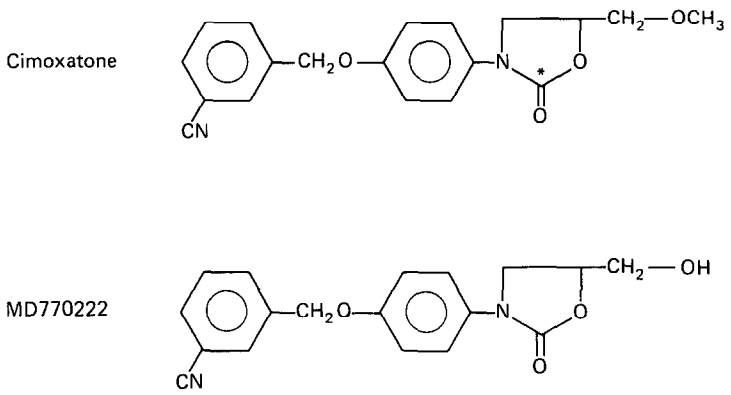


Fig. 1. Structures of cimoxatone and its plasma metabolite MD 770222. *^{14}C label.

FFA/mole of HSA. In a preliminary experiment, binding to HSA II was investigated with cimoxatone concentrations of 0.1 mg/l. (0.3 μM) and 5 mg/l. (15 μM) vs concentrations of HSA II ranging from 0.5 g/l. (7 μM) to 40 g/l. (580 μM). For binding experiments, HSA solutions of 2 g/l. (29 μM, HSA I and HSA II) and 40 g/l. (580 μM, HSA II) in phosphate buffer were prepared.

(b) *Binding to total plasma proteins*: Plasma protein binding was studied *in vitro* using fresh heparinized plasma from human volunteers; buffer solutions were prepared with [^{14}C]cimoxatone in order to obtain concentrations ranging from 0.05 mg/l. (0.15 μM) to 70 mg/l. (207 μM).

(c) *Binding to other isolated plasma proteins*: The binding of cimoxatone to isolated plasma protein fractions was investigated. Solutions of each protein in phosphate buffer, at a physiological concentration, were prepared: α_1-acid glycoprotein (α_1-AGP, Sigma) 0.9 g/l., γ-globulins (γ-G, Sigma HG II) 15 g/l., very low density lipoproteins (VLDL) 1.4 g/l., low density lipoproteins (LDL) 3.3 g/l. and high density lipoproteins (HDL) 4.3 g/l. The cimoxatone concentration was 3 μM for the α_1-AGP experiment, and was increased from 0.1 to 70 mg/l. (0.3–207 μM) for the γ-G and lipoprotein experiments.

VLDL, LDL and HDL fractions were obtained from fresh human serum using a differential ultracentrifugation method [8]. The protein content of each fraction was estimated by the method of Lowry *et al.* [9]; the concentrations of lipoproteins were calculated assuming that the apoprotein corresponded to about 10% (w/w) of VLDL, 20% of LDL and 50% of HDL [10].

2. *Interactions*. The influence of bilirubine (Sigma), L-tryptophan (Sigma), flunitrazepam (Roche), oxazepam (Wyeth), imipramine (Ciba-Geigy), aspirin, phenylbutazone (Sigma) and warfarin (Sigma) on the binding of cimoxatone to HSA II (29 and 580 μM) was investigated. Displacement studies of cimoxatone from HSA II, 29 μM and 580 μM, were also conducted with MD 770222.

3. *Calculation of binding parameters*. The percentages of cimoxatone (from 0.15 to 207 μM) bound to HSA I and HSA II (29 μM) were analysed in order to obtain the number of drug binding sites (n) on the albumin molecule, and the apparent association constant (K) of the drug for these sites.

The number of moles of drug bound per mole of albumin $[B]/[C_p]$ was calculated and plotted as the ordinate against the number of unbound moles of drug $[F]$. From these data, n and K were calculated from a non-linear least-squares fit using a Gauss–Newton algorithm with a Tektronix 4052 desk computer and the equation:

$$[B]/[C_p] = nK[F]/(1 + K[F]).$$

This plot is based on a linear transformation of $[B]$ and $[F]$ and allows a better estimation of n and K if compared with a Scatchard plot, where the accuracy of the $[B]/[F]$ values is affected at the high free ligand concentrations [11].

RESULTS

1. Binding of cimoxatone to HSA and plasma proteins

(a) Determination of binding parameters to HSA. For a given concentration of cimoxatone (0.3 and 15 μM), and increasing concentrations of HSA II (Fig. 2), the binding of cimoxatone to HSA II was saturable. The percentage of cimoxatone bound reached a plateau at the higher concentrations of HSA II. A HSA II concentration of 29 μM, which

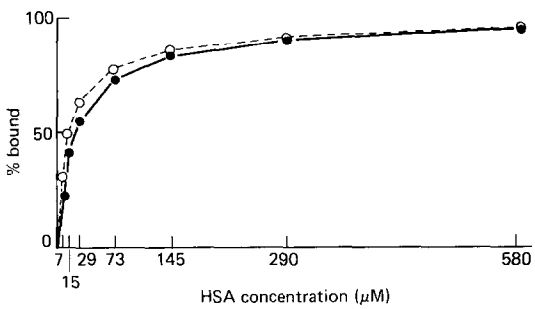


Fig. 2. Cimoxatone binding to HSA II (7–580 μM). Cimoxatone 0.3 μM, O---O; cimoxatone 15 μM, ●——●.

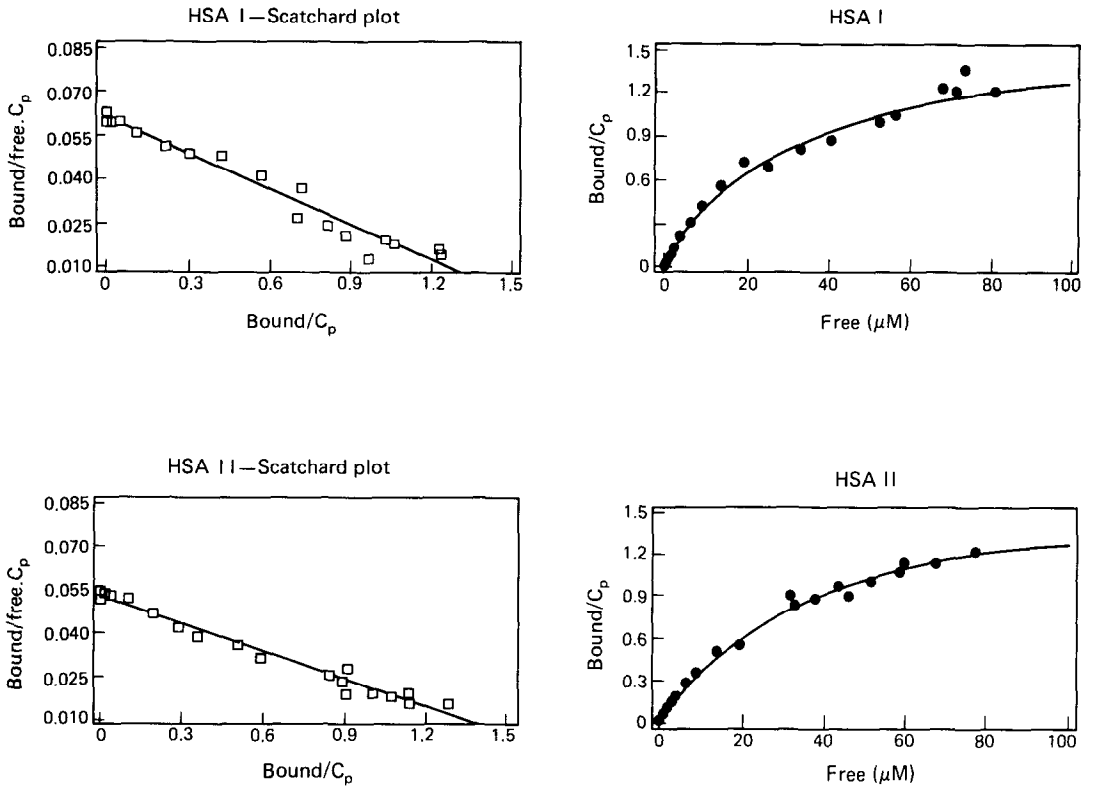


Fig. 3. Binding of cimoxatone (0.15–207 μM) to HSA I and HSA II (29 μM). Scatchard plots and non-linear least-squares fits.

is in the saturable part of the curve, was chosen for the determination of the binding parameters.

The Scatchard plots (Fig. 3) showed a single class of binding sites to HSA I and to HSA II, 29 μM. Cimoxatone binding was studied over the range of 0.15–207 μM; non-linear least-squares fits (Gauss–Newton algorithm) of the curves $[B]/[C_p]$ vs $[F]$ for HSA I and HSA II, 29 μM, binding are shown in Fig. 3.

Within the range of concentrations studied, the binding percentage decreased from 60 to 31% for HSA II, and from 63 to 33% for HSA I. The affinity constant and number of binding sites were similar for HSA II and HSA I binding:
$K = (2.89 \pm 0.25) \times 10^4\ M^{-1}$; $n = 1.72 \pm 0.07$ for HSA II
$K = (2.99 \pm 0.46) \times 10^4\ M^{-1}$; $n = 1.77 \pm 0.11$ for HSA I.

(b) *Binding to HSA* II *and to plasma proteins.* For drug concentrations ranging from 0.15 to 207 μM, the binding of cimoxatone to HSA II (580 μM) and to total plasma proteins was not saturable (Table 1). The binding percentage was 93 ± 1% ($n = 29$) for HSA II, and 94 ± 2% ($n = 25$) for total plasma proteins. These results suggest that cimoxatone binds preferentially to albumin in plasma.

As shown in Table 1 and Fig. 4, when compared with the amount bound to albumin, cimoxatone was only moderately bound to the other isolated protein fractions: 12 ± 2% ($n = 5$) was bound to α_1-AGP; and only 7 ± 1% ($n = 22$) was bound to γ-G. Binding to lipoproteins was: 10 ± 2% ($n = 28$) to VLDL;

Table 1. Binding of cimoxatone to plasma protein fractions (mean values and range, $n = 4$)

	Protein concentration		Cimoxatone concentration		% of cimoxatone bound	
	g/l	μM	mg/l	μM	Mean	Range
Plasma	70	—	0.05–70	0.15–207	95	93–96
HSA II	40	580	0.10–70	0.30–207	93	93–94
α_1-AGP	0.9	22	1	3	12	11–15
VLDL	1.4	0.4	0.10–70	0.30–207	10	7–13
LDL	3.3	1.3	0.10–70	0.30–207	22	21–23
HDL	4.3	10–22	0.10–70	0.30–207	50	49–53
γ-G	15	16–100	0.10–70	0.30–207	7	5–8

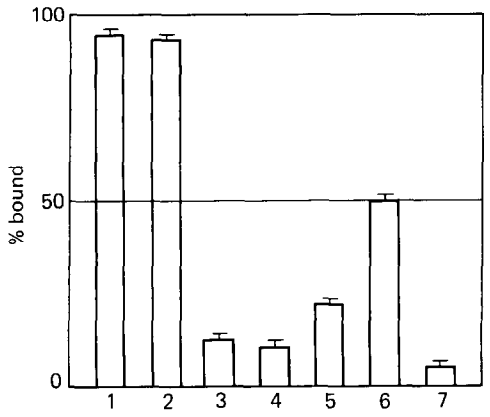
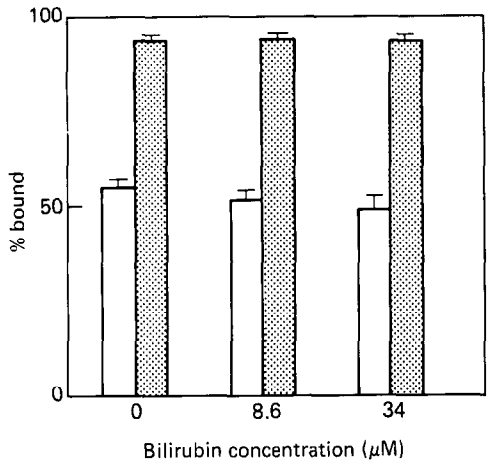


Fig. 4. Binding of cimoxatone to plasma protein fractions. (1) Plasma, (2) HSA II 40 g/l., (3) α_1-AGP 0.9 g/l., (4) VLDL 1.4 g/l., (5) LDL 3.3 g/l., (6) HDL 4.3 g/l., (7) γ-G 15 g/l. Mean values ± S.D.



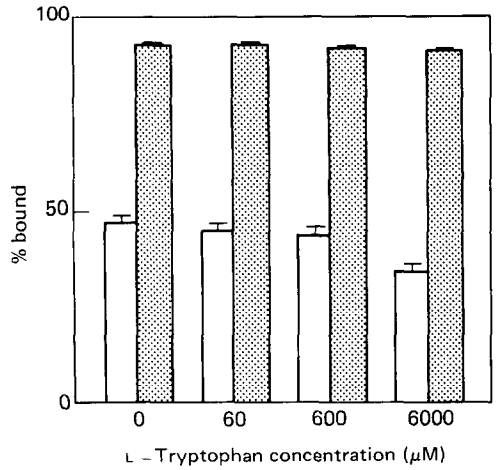


Fig. 5. Effect of bilirubin on the binding of 30 μM cimoxatone to HSA II, 29 μM (□) and 580 μM (▨). Effect of L-tryptophan on the binding of 60 μM cimoxatone to HSA II, 29 μM (□) and 580 μM (▨).

22 ± 1% (n = 29) to LDL; and 50 ± 1% (n = 29) to HDL.

2. *Interactions with bilirubin and* L-*tryptophan*

As shown in Fig. 5, the binding of cimoxatone (30 μM) to 580 μM HSA II was not modified when bilirubin was added to the HSA solution at physiological (8.6 μM) and pathological (34 μM) concentrations. However, at HSA II concentrations of 29 μM, the binding was slightly decreased for a bilirubin concentration of 34 μM. Interaction with L-tryptophan for binding to HSA II (29 μM) was found only at the highest concentration studied (6000 μM) (Fig. 5); no interaction was found for L-tryptophan concentrations of 60 and 600 μM. Binding to 580 μM HSA II was not modified by L-tryptophan at concentrations of 60 μM (physiological concentration), 600 and 6000 μM.

3. *Interactions with other drugs*

As shown in Fig. 6, the binding of cimoxatone (3 μM) to HSA II (29 μM) was not modified by flunitrazepam, oxazepam, imipramine and aspirin for concentrations of these drugs even higher than the therapeutic ones. Displacement was found with warfarin and phenylbutazone (Fig. 7) at a HSA II concentration of 29 μM, but no interaction occurred at 580 μM.

4. *Interactions with the metabolite MD* 770222

MD 770222 displaced cimoxatone from 29 μM HSA II as shown in Table 2. The binding percentage of cimoxatone (3 μM) decreased from 61 to 52% at metabolite concentrations ranging from 0.15 to 61.7 μM. This suggests that both compounds share common binding sites on HSA II. However, when the experiment was conducted with 580 μM HSA II and 3 μM MD 770222, the binding percentage of cimoxatone (0.15–15 μM) was 94% and was not modified by the metabolite.

DISCUSSION

The binding parameters of cimoxatone to 29 μM HSA II have been determined in a concentration range from 0.15 to 207 μM. The solubility in buffer did not allow cimoxatone concentrations to increase above 207 μM. Within this range, cimoxatone showed one class of binding sites to HSA (2 sites per mole of protein), with a moderate affinity, $K = 2.9\text{–}3 \times 10^4\,M^{-1}$. The percentage bound to whole plasma and to 580 μM HSA II was independent of cimoxatone concentration up to 207 μM (Table 1). No evidence of saturation occurred up to this concentration, and the binding percentage to HSA and to total plasma proteins was 93–96%. These results indicate that although cimoxatone was bound to lipoprotein fractions *in vitro*, it is likely to bind preferentially to HSA in plasma. In spite of a high degree of binding to HSA, no displacement of cimoxatone was found with concentrations of metabolite expected during repeated administration of cimoxatone [12].

For 29 μM HSA II, a small interaction occurred with high concentrations of the metabolite, which could indicate common binding sites on the HSA

Table 2. Effect of MD 770222 (0.15–61.7 μM) on the binding of 3 μM cimoxatone to 29 μM albumin (mean values and range, $n = 4$)

MD 770222 concentration		% of cimoxatone bound	
mg/l	μM	Mean	Range
0	0	61	59–63
0.05	0.15	61	59–62
0.1	0.31	60	58–62
0.25	0.77	58	57–60
1.0	3.10	58	58–58
2.0	6.2	57	56–58
5.0	15.4	56	56–57
20.0	61.7	52	52–53

molecule for both compounds (Table 2). The binding parameters were the same when HSA I containing 3.45 mole of FFA per mole of HSA was used. A FFA/albumin ratio of 3.45 corresponds to a serum FFA concentration of about 2 meq/l. Normal FFA concentrations in human plasma range between 0.2 and 0.5 meq/l. Although many drugs are not affected by changes in FFA concentration, fatty acids have allosteric effects on albumin [13] which can result in increases or decreases in binding at specific sites: phenytoin [14], indomethacin [11] and digitalis glycosides [15] are displaced from albumin by FFA. Endogenous compounds such as bilirubin and L-tryptophan were also unable to displace cimoxatone from its binding sites on the HSA molecule (580 μM). For 29 μM HSA, a slight decrease of the percentage bound occurred in presence of 34 μM bilirubin, and with L-tryptophan when the molar ratio L-tryptophan/cimoxatone approached 100.

Bilirubin interacts with one high affinity binding site, $K = 10^8 M^{-1}$, which also binds acidic drugs [16]. L-Tryptophan interacts at a single site, with $K = 6.7 \times 10^3$ to $1.6 \times 10^4 M^{-1}$, but binding is less specific than for the bilirubin site [17]. Competition has been demonstrated for a wide variety of compounds including salicylates [18], clofibrate [18], fatty acids [19] and benzodiazepines [20].

Among the drugs studied over a large range of concentrations, only warfarin and phenylbutazone displaced cimoxatone from the albumin binding sites. These two drugs bind strongly to the same site of the HSA molecule, and displace each other [21]. The present results suggest that warfarin and phenylbutazone compete for the same binding sites as cimoxatone on the HSA molecule. Phenylbutazone is more strongly bound than warfarin to HSA [22, 23], which could explain its higher displacement effect compared to that of warfarin.

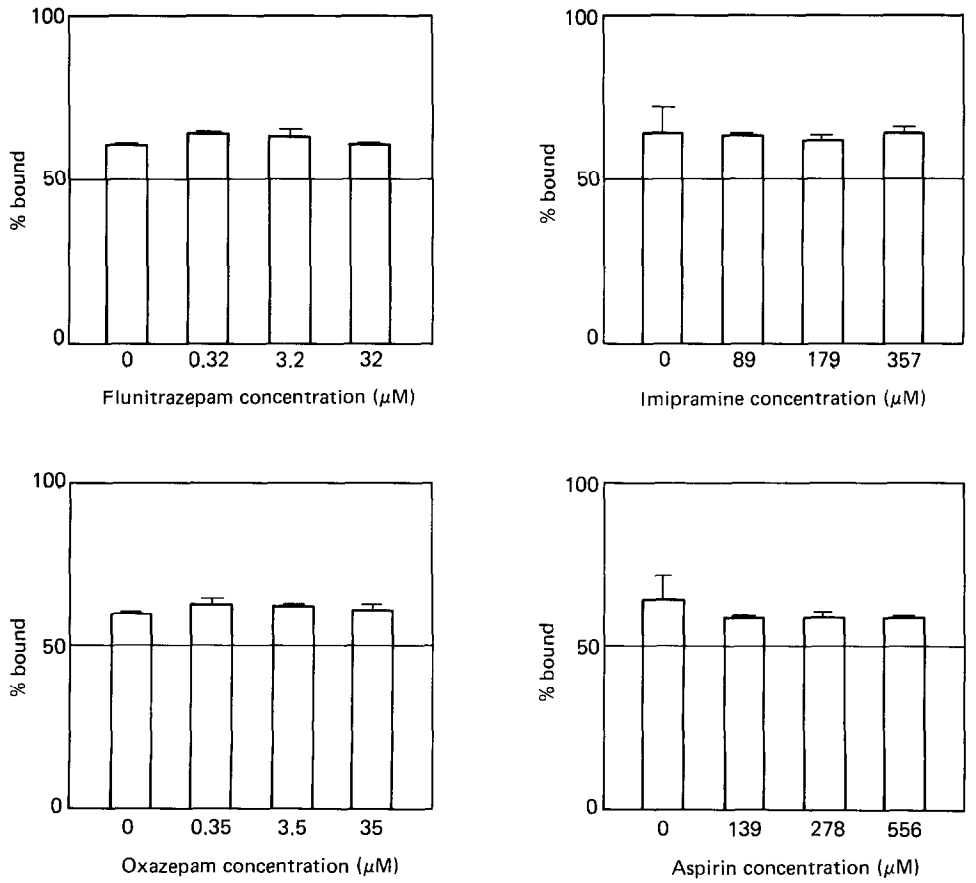


Fig. 6. Effect of imipramine, aspirin, oxazepam and flunitrazepam on the binding of 3 μM cimoxatone to 29 μM HSA II.

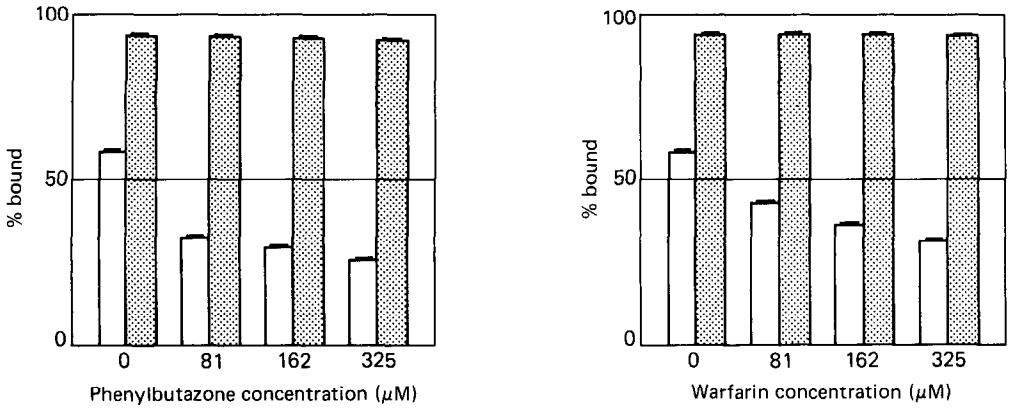


Fig. 7. Effect of warfarin and phenylbutazone on the binding of 3 μM cimoxatone to HSA II, 29 μM (□) and 580 μM (▨).

However, the interaction between cimoxatone and warfarin or phenylbutazone does not occur for the 580 μM concentration of HSA II. The other drugs studied (imipramine, aspirin, oxazepam and flunitrazepam) did not cause a significant change in per cent binding of cimoxatone. This lack of effect would suggest that these compounds interact with albumin at sites different from those of cimoxatone. Imipramine has been shown to bind to a greater number of albumin binding sites with a weak association constant [24]. Acetylsalicylic acid acetylates HSA, which can result in an increased or decreased affinity of other ligands [25]. Cimoxatone is likely to bind to the warfarin binding site, which is probably different from the binding site for benzodiazepines [26].

In conclusion, cimoxatone is bound with a moderate affinity to two sites of the albumin molecule. No changes in the free active fraction of cimoxatone, due to variations in binding at different doses, or in the presence of other drugs or endogenous ligands, should occur during its clinical use.

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