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EFFECT OF β-CARBOLINE DERIVATIVES ON THE BINDING OF L-TRYPTOPHAN AND DIAZEPAM TO BOVINE AND HUMAN ALBUMIN

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Abstract—The effects of 12 β-carboline derivatives on the binding of L-tryptophan and diazepam to bovine and human albumin have been investigated to seek similarities between the indole binding site on albumin and the benzodiazepine receptor in the brain. The binding of L-tryptophan and diazepam was measured at 37° and pH 7.4 by equilibrium dialysis. Norharmane was the most potent inhibitor of the binding of L-tryptophan and diazepam to both bovine and human albumin. The kinetics of the inhibitory effects of several of the β-carbolines were studied. Norharmane decreased the value (n) for the number of binding sites for the binding of L-tryptophan to both bovine and human albumin. Norharmane and harmane decreased the apparent association (K_a) but increased n for the interaction of diazepam with bovine albumin. Norharmane also had a similar effect on the binding of diazepam to human albumin. The similarities between the inhibitory effects of the β-carbolines on the binding of L-tryptophan and diazepam to albumin and the affinity of the β-carbolines for the central benzodiazepine receptor point to some common structural requirements for binding to the receptor and to albumin.

Competitive displacement of one ligand by another from a protein is thought to be evidence for binding to the same region on that protein [1]. L-Tryptophan and diazepam have been shown to bind to the same region on human albumin, namely the indole binding site [2] or site II [3] by competitive inhibition [4–6]. L-Tryptophan has also been shown to displace diazepam and other benzodiazepines from benzodiazepine receptors on rat brain synaptosomes [7]. The endogenous ligand for the benzodiazepine receptor has not yet been identified but it may be related in structure to the β-carbolines. In particular, β-carboline-3-carboxylic-acid-ethyl-ester (β-CCE) has been shown to possess high affinity for the benzodiazepine receptor and to displace benzodiazepines from this site [8]. The binding of a series of β-carbolines (Fig. 1) to the indole binding site has therefore been investigated to establish whether any similarities exist between the indole binding site on albumin and the benzodiazepine receptor in the brain.

MATERIALS AND METHODS

Materials. Bovine albumin, fatty acid free (fraction V; A7030; batch no. 123F-0573), human albumin (fraction V; A1653; batch no. 44F-9359) and the following β-carboline derivatives were obtained from the Sigma Chemical Co. (Poole, U.K.): norharmane, harmane, harmaline, harmine, harmalol and harmol (supplied as hydrochloride salts), 6-methoxy-harmane, 6-methoxy-harmalan and 1-methyl-tetrahydro-β-carboline-3-carboxylic acid (supplied as the free base). β-Carboline-3-carboxylic-acid-ethyl-ester (β-CCE; lot no. JHG-v-3) was obtained from Research Biochemicals Inc. (Wayland, MO, U.S.A.). Tetrahydro-β-carboline and 1-methyl-tetrahydro-β-carboline (HCl salts) were kindly supplied by Dr O. Beck, Stanford University Medical Centre, UCLA, U.S.A. Diazepam (Ro 05-2807) was a kind gift from Roche Products Ltd (Welwyn Garden City, U.K.). Unlabelled L-tryptophan ("AnalaR" grade) was obtained from BDH Chemicals Ltd (Speke, Liverpool, U.K.).

The following radiochemicals were obtained from Amersham International plc (Amersham, U.K.): L-[methylene-^{14}C]tryptophan (sp. act. 53.5 mCi/mmol) and [2-^{14}C]diazepam (54 mCi/mmol). All other reagents were of "AnalaR" grade and were obtained from BDH Chemicals Ltd. Visking tubing (size 9-36/32", flat width 1.25 in.) was obtained from Medicell International Ltd (London, U.K.).

Displacement of L-tryptophan and diazepam from albumin. Stock solutions of L-tryptophan (450 μM) were prepared in phosphate buffer pH 7.4 containing sodium chloride (5.75 g/L Na_2HPO_4 (40 mM), 1.50 g/L $NaH_2PO_4 \cdot 2H_2O$ (9.6 mM), 2.08 g/L NaCl (0.04 mM)); [9]. Diazepam stock solutions (3.6 mM) were prepared in 25% (v/v) ethanol/water. Radiolabelled ligand was then added to the stock solution to give a concentration of radioactivity of 0.025 μCi/mL.

Solutions of bovine and human albumin (3 g/100 mL, 450 μM) were prepared in phosphate buffer pH 7.4. Human albumin was defatted prior to use with activated charcoal at pH 3.0 [10] and diluted to 3 g/100 mL (450 μM) with buffer. Stock solutions of 1.8 mM were prepared for all displacing agents (β-carboline derivatives, L-tryptophan and diazepam) and were diluted with phosphate buffer pH 7.4 to

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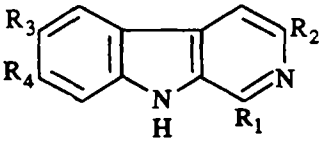
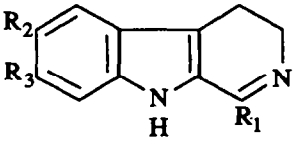
β-Carbolines



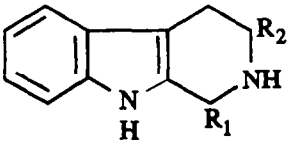
	R_1	R_2	R_3	R_4
Norharmane	H	H	H	H
Harmane	CH_3	H	H	H
β-CCE*	H	$CO_2CH_2CH_3$	H	H
6-Methoxy-harmane	CH_3	H	OCH_3	H
Harmol	CH_3	H	H	OH
Harmine	CH_3	H	H	CH_3

Dihydro-β-carbolines



	R_1	R_2	R_3
6-Methoxy-harmalan	CH_3	OCH_3	H
Harmalol	CH_3	H	OH
Harmaline	CH_3	H	OCH_3

Tetrahydro-β-carbolines



	R_1	R_2
Tetrahydro-β-carboline	H	H
1-Methyl-tetrahydro-β-carboline	CH_3	H
1-Methyl-tetrahydro-β-carboline-3-carboxylic acid	CH_3	CO_2H

* β-Carboline-3-carboxylic acid ethyl ester

Fig. 1. Structures of the β-carboline derivatives.

give concentrations of 900 and 450 μM. Equal volumes of ligand, albumin and inhibitor or buffer were mixed and incubated at room temperature for 20 min. The binding of L-tryptophan and diazepam to albumin was measured by equilibrium dialysis with a Dianorm apparatus (Diachema AG, Zurich [11]). Half-cell teflon compartments of 1 mL capacity were used. Samples (0.9 mL) were dialysed against an equal volume of phosphate buffer at 37°, pH 7.4. The final concentration of ethanol was 1% (v/v) in solutions containing diazepam and 4% (v/v) in solutions containing β-CCE as a displacer. Control experiments to measure the binding of L-tryptophan and diazepam to albumin were done in the presence of 4% (v/v) ethanol/buffer when β-CCE was used as a displacer.

After dialysis, samples (0.5 mL) were taken from each side of the dialysis membrane and mixed with scintillation fluid (4.5 mL) for counting in a Packard Tricarb 4640 scintillation counter. Automatic quench correction was performed by the external standard channels-ratio method with [^{14}C]*n*-hexadecane as the internal standard.

Kinetics of L-tryptophan and diazepam displacement from albumin. The kinetics of norharmane, harmane and β-CCE displacement of L-tryptophan (10–150 μM) from bovine and human albumin (1 g/100 mL, 150 μM) were investigated by equilibrium dialysis. The kinetics of displacement of diazepam from bovine and human albumin by norharmane and harmane were investigated in the same way. The concentrations of total and unbound L-tryptophan and diazepam were measured by scintillation counting and the number of binding sites (n) and apparent association constant (K_a) were obtained by fitting the data with the program LIGAND developed by Munson and Rodbard [12]. This program calculates the parameters n and K_a, together with their standard errors.

RESULTS

Inhibition of L-tryptophan binding to albumin

Figure 1 shows the structure of the β-carboline derivatives used in this study. Norharmane was the most potent inhibitor of L-tryptophan binding to bovine albumin (Table 1) and was followed in order of decreasing potency by harmane, β-CCE and 6-methoxy-harmane. Harmol, harmine, harmaline, harmalol, 6-methoxy-harmalan, 1-methyl-tetrahydro-β-carboline and 1-methyl-tetrahydro-β-carboline-3-carboxylic acid all tended to increase L-tryptophan binding. This effect was not concentration-dependent, however, and because the decrease in percentage unbound was less than 2% in all cases the results are not tabulated.

Norharmane was also the most potent inhibitor of L-tryptophan binding to charcoal-extracted human albumin (Table 1) and it was followed in order of decreasing potency by β-CCE and harmane. This order was similar to that found with bovine albumin. Diazepam and L-tryptophan had a mutually inhibitory action: that is, diazepam inhibited the binding of L-tryptophan and *vice versa* (Table 2). This result would be expected if the two ligands interacted with a common area on albumin.

Table 1. Unbound concentrations of [^{14}C]L-tryptophan (initial total concentration 150 μM) in the presence of either bovine albumin (150 μM) or charcoal-extracted human albumin (150 μM) and a range of concentrations of β-carboline derivatives

	Concentration of β-carboline derivative (μM)			
β-Carboline derivative	0	150	300	600
Bovine albumin				
Norharmane	51*	63	68	70
Harmane	51	56	59	62
β-CCE	53	56	60	60
6-Methoxy-harmane	54	52	54	59
Human albumin				
Norharmane	52†	67	68	73
β-CCE	58	59	60	66
Harmane	50	49	53	55

* Unbound concentration of L-tryptophan (μM); mean of five measurements, SD less than 1 μM in each case.

† SD less than 2 μM in each case in the presence of human albumin.

Table 2. Unbound concentrations of [^{14}C]L-tryptophan (initial total concentration 150 μM) and [^{14}C]diazepam (initial concentration 150 μM) after displacement from bovine albumin (150 μM) by diazepam and L-tryptophan, respectively

	Concentration of displacer (μM)			
Ligand	0	150	300	600
L-Tryptophan	50*	52	56	61
Diazepam	37	37	39	42

* Unbound concentration of L-tryptophan or diazepam (μM); mean of five measurements; SD less than 2 μM in each case.

Inhibition of diazepam binding to albumin

Norharmane was the most potent inhibitor of diazepam binding to bovine albumin (Table 3) and was followed in order of decreasing potency by harmane, 1-methyl-tetrahydro-β-carboline-3-carboxylic acid, 6-methoxy-harmane and harmol. Harmalol and 1-methyl-tetrahydro-β-carboline slightly increased diazepam binding but the effect was not concentration dependent and the decrease in percentage unbound was less than 2% in each case. Harmaline, harmine, 6-methoxy-harmalan, tetrahydro-β-carboline and β-CCE did not change diazepam binding to bovine albumin (results not shown).

Norharmane was also the most potent inhibitor of diazepam binding to charcoal-extracted human albumin (Table 3). 1-Methyl-tetrahydro-β-carboline-3-carboxylic acid was the next most potent inhibitor, while harmane did not alter the binding of diazepam to human albumin. These results are similar to those found with L-tryptophan, where the second and third

Table 3. Unbound concentrations of [^{14}C]diazepam (initial total concentration 150 μM) in the presence of either bovine albumin (150 μM) or charcoal-extracted human albumin (150 μM) and a range of concentrations of β-carboline derivatives

β-Carboline derivative	Concentration of β-carboline derivative (μM)			
	0	150	300	600
Bovine albumin				
Norharmane	38*	44	45	46
Harmane	34	37	38	40
6-Methoxy-harmane	33	34	34	35
Harmol	33	34	36	33
1-Methyl tetrahydro-β-carboline-3-carboxylic acid	33	34	36	36
Human albumin				
Norharmane	14†	16	20	22
1-Methyl tetrahydro-β-carboline-3-carboxylic acid	15	18	18	20
Harmane	15	15	15	15

* Unbound concentration of diazepam (μM); mean of five measurements; SD less than 1 μM in each case.
† SD less than 2 μM in each case in the presence of human albumin.

Table 4. Effect of norharmane, harmane and β-CCE on the binding parameters n and K_a of [^{14}C]L-tryptophan (10, 20, 30, 50, 75 and 150 μM; three replicates in each case) to bovine albumin (150 μM)

Inhibitor concn. (μM)	n	$10^{-4} \times K_a$ (L/mol)
Control	0.63 ± 0.03	1.75 ± 0.10
Norharmane		
100	0.38 ± 0.04	1.60 ± 0.25
125	0.33 ± 0.01	1.38 ± 0.06
Harmane		
100	0.64 ± 0.09	1.15 ± 0.20
125	0.61 ± 0.03	1.53 ± 0.11
β-CCE		
150	1.18 ± 0.53	0.38 ± 0.18

Estimates of n, K_a and the SE were computed with the program LIGAND.

Table 5. Effect of norharmane, harmane and β-CCE on the binding parameters of [^{14}C]L-tryptophan (10, 20, 30, 50, 75 and 150 μM; three replicates in each case) to charcoal-extracted human albumin (150 μM)

Inhibitor concn. (μM)	n	$10^{-4} \times K_a$ (L/mol)
Control	0.63 ± 0.28	1.17 ± 0.58
Norharmane		
100	0.38 ± 0.03	1.19 ± 0.98
125	0.58 ± 0.08	0.99 ± 0.17
Harmane		
100	1.02 ± 0.21	0.84 ± 0.20
125	1.07 ± 0.13	0.92 ± 0.13
β-CCE		
150	0.63 ± 0.07	0.95 ± 0.13

Estimates of n, K_a and the SE were computed with the program LIGAND.

most potent displacers of ligand from bovine albumin reverse places when tested against human albumin.

Kinetics of L-tryptophan and diazepam displacement from albumin

The inhibitory effects of the β-carbolines on the binding of L-tryptophan and diazepam to human and bovine albumin were generally of a mixed type (Tables 4–6). The effects on parameter K_a were similar with both proteins and the K_a was reduced in all cases but one, that was the K_a for the binding of L-tryptophan to human albumin which was unchanged by the presence of norharmane at 100 μM (Table 5). The effects of the β-carbolines on n were inconsistent from bovine to human albumin and an increase, decrease and no change in this parameter were observed (Tables 4–6).

Kinetics of diazepam displacement from albumin

Norharmane and harmane (125 μM) decreased

Table 6. Effect of norharmane and harmane on the binding parameters n and K_a of [^{14}C]diazepam (50, 75, 100, 150, 175, 200 and 250 μM; three replicates in each case) to bovine albumin (BSA; 150 μM) and human albumin (HSA; 150 μM).

Albumin	Inhibitor concn. (μM)	n	$10^{-3} \times K_a$ (L/mol)
BSA	Control	1.6 ± 0.4	8.55 ± 0.34
	Norharmane		
	125	17.3 ± 16.2	0.50 ± 0.53
	Harmane		
	125	10.1 ± 4.0	0.97 ± 0.42
HSA	Control	1.4 ± 0.1	50.28 ± 5.57
	Norharmane		
	125	1.8 ± 0.1	33.69 ± 4.61

Estimates of n, K_a and the SE were computed with the program LIGAND.

the K_a (affinity) but increased the value of n for the binding of diazepam to bovine albumin (Table 6). The same effect was observed when norharmane (125 μM) displaced diazepam from charcoal extracted human albumin (Table 6).

DISCUSSION

The indole binding site on albumin and the central benzodiazepine receptor have similar requirements for high affinity ligand binding. It should be noted, however, that the affinities can differ by several orders of magnitude between the two different contexts of interaction with albumin, the potential transport protein, and the presumably specific benzodiazepine tissue receptors which have the higher affinity. The indole binding site on albumin is thought to be a hydrophobic cleft 12 to 16 Å deep and 6 to 8 Å wide [13]. The indole ring structure of L-tryptophan could interact with the hydrophobic site, while the uncharged amino group of the side chain may be associated with a positively charged amino group within the binding site [14]. The central benzodiazepine receptor is also thought to be a planar cleft [15–17]. The specific interaction of the nitrogen atom of the β-carboline ring structure with a nearby cationic receptor site is considered to be important for high affinity binding, rather than hydrophobic interactions of the ring structure with the receptor [16, 18]. Hydrogen bonding of an ester or nitrile group on the C_3 side-chain with the benzodiazepine receptor may also be important for high affinity binding [16–19]. Binding to the central benzodiazepine receptor is inhibited by substitution at positions C_1, C_7 and N_9 and by saturation or partial saturation of the β-carboline ring structure [15–17, 20]. The results presented here show the same to be true for β-carboline binding to the indole binding site on albumin.

The similar structure–activity relationships of the central benzodiazepine receptor and the indole binding site on albumin is reflected in the similar orders of potency for β-carboline binding to the two sites. The order of potency for displacement of L-tryptophan from human albumin was norharmane > β-CCE > harmane > 6-methoxy-harmane. The order for displacement of diazepam from human albumin was norharmane > 1-methyl-tetrahydro-β-carboline-3-carboxylic acid > harmane > 6-methoxy-harmane. Skolnick *et al.* [21] found the order of potency for displacement of [^{3}H]flunitrazepam from central benzodiazepine receptors to be β-CCE ≫ norharmane > harmane ≫ tetrahydro-β-carbolines. So far the possibility of sequence homology between albumin and the α subunit of the GABA receptor, the presumed binding site for the benzodiazepines, has not been investigated. Albumin is not normally extensively glycosylated whereas there is believed to be a total of 10 potential glycosylation sites per receptor complex [22].

The minor differences in order at the indole binding site and at the central benzodiazepine receptor may be explained by the different temperatures at which the experiments were carried out. Binding experiments performed *in vitro* are temperature dependent, and binding is reduced as temperature is increased [23]. Receptor binding studies are usually carried out between 0° and 4° [15, 16, 19] while equilibrium dialysis was performed at 37° throughout this study.

The kinetics of L-tryptophan and diazepam displacement from albumin were of a mixed type, that is both competitive and non-competitive displacement were present [24]. The reduced affinity of L-tryptophan and diazepam for albumin in the presence of norharmane indicated that the β-carboline acted directly at the indole binding site (competitive displacement). Changes in the value of n for the ligands, however, pointed to a conformational change in albumin in the presence of norharmane (non-competitive displacement). It is possible that norharmane and the β-carboline derivatives have affinity for other specific sites on albumin. Binding to these areas could change the tertiary structure of albumin, and so affect L-tryptophan and diazepam binding indirectly. Such potential sites are those for salicylate and phenol red on albumin because it has been shown that the indole binding site interacts with both these areas [6]. The warfarin site (site I; [3]) could also accept a heterocyclic ring structure with a delocalised negative charge such as norharmane; however, ligand interaction with the warfarin site has been shown not to affect binding at the indole site on albumin [3, 6, 25].

In conclusion, of all the β-carboline derivatives tested, norharmane was the most potent inhibitor of the binding of L-tryptophan and diazepam to the indole site on albumin. The inhibitory potency of the β-carboline derivatives presumably reflects their affinity for albumin and, on the basis of this assumption, the most highly bound β-carboline derivatives are norharmane, harmane, β-CCE and 1-methyl-tetrahydro-β-carboline-3-carboxylic acid. The kinetics of displacement showed that the reduction in binding of L-tryptophan and diazepam could be caused by occupation of the indole site and also by some conformational changes of albumin due to binding of the β-carboline derivatives elsewhere on the protein. Kragh–Hansen [6, 24, 26] has suggested that mixed kinetics of displacement could be caused by binding of ligands to two sites with mutual interactions. In view of this, further work with β-carboline derivatives at the salicylate site and the phenol red site may help to explain the mixed kinetics of displacement of L-tryptophan and diazepam from albumin. Norharmane has been used as an inhibitor to modulate the plasma concentration of unbound L-tryptophan in experiments to investigate the relationship between unbound concentration and brain uptake in the rat *in vivo* [27]. It is therefore possible that at least some of the pharmacological activity of the β-carbolines may derive from the displacement of an endogenous ligand for the benzodiazepine receptor from plasma and tissue binding sites.

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