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INTERACTION OF BENZODIAZEPINE DERIVATIVES WITH BOVINE SERUM ALBUMIN—I

GEL FILTRATION STUDIES*†

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Abstract—The binding of eleven benzodiazepine derivatives to bovine serum albumin was determined by means of Sephadex gel filtration. The albumin binding of the substances was characterized by the percentage of drug bound, the binding constants k^+, K_1, and m, the number of binding sites per albumin molecule, and the free binding energy. The binding of the benzodiazepines to bovine serum albumin (BSA) is discussed in respect to the binding of these drugs to human serum albumin (HSA). The following great differences in the binding behaviour of both albumins for the benzodiazepines have been found: (1) The affinities of the binding of most of the drugs to BSA are exceptionally smaller than those to HSA. (2) Two benzodiazepines, lorazepam and clonazepam, are bound to BSA in a higher extent than to HSA. (3) Most of the benzodiazepines have two or three binding sites on the BSA molecule, in contrast to the single binding site on the HSA molecule. The binding of the benzodiazepines to BSA is positively influenced by the pH-value of the solution in a similar way as found for HSA. The benzodiazepines are the first group of drugs known in which binding to both albumins differs so fundamentally. The reason for these large differences and their pharmacological significance are discussed.

It was demonstrated in previous investigations that the benzodiazepines are bound to human serum albumin (HSA) in a very specific manner[2, 3], and that the binding to HSA differs in some aspects from the binding behaviour of other substances to HSA. The binding of the benzodiazepines is influenced in an unusual way by the substituents of the molecule[2] and does not depend on the hydrophobic nature of the ligands[2]. Furthermore, the binding is highly stereospecific in an until now unknown degree[4]. In contrast to the high stereospecific binding of oxazepam hemisuccinate to HSA[4], the stereospecificity of the binding of this drug to bovine serum albumin (BSA) is much smaller[5] and the *d*-isomer of this drug was found to have a more than eight-fold smaller affinity to BSA than to HSA[5].

Because these differences with both albumins were exceptionally high, we tried to investigate if they were due to stereochemical aspects alone, or if BSA has a generally different binding behaviour for the benzodiazepine derivatives. This would be of interest, since there is little information on greater differences in the binding of small ligands to both albumins. It is known that the binding of ligands to HSA and BSA can differ[7–12], but in most cases these differences are relatively small[6–9] and within a group of substances the increase or decrease of the affinities is similar for both albumins[8, 11, 12].

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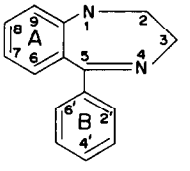
MATERIALS AND METHODS

Materials. Bovine serum albumin (BSA) was obtained from Behringwerke, Marburg (quality: "trocken, reinst"), electrophoretic purity 100%. All drugs were obtained from the manufacturers: Bromazepam, chlordiazepoxid, clonazepam, demoxepam, diazepam, flurazepam, and nitrazepam from Hoffmann–La Roche AG, Grenzach; oxazepam from Boehringer, Ingelheim; dipotassium clorazepate from Mack, Illertissen, lorazepam from Wyeth, Münster; tetrazepam from Clin-Byla, Paris. The formula of the 2,3-dihydro-5-phenyl-1,4-benzodiazepine molecule is shown below. For the chemical formulas of the derivatives see Ref. 13.



All other chemicals were of reagent grade. All solutions were made with deionized water.

Albumin binding measurements. The binding of the benzodiazepine derivatives to BSA was studied by the gel filtration technique of Krieglstein and Kuschinsky[14]. The suitability of this method to the study of protein binding of benzodiazepine derivatives was discussed elsewhere[2]. The experiments were performed on 20 × 1·2 cm columns of Sephadex G-50 fine (Pharmacia, Uppsala) at room temperature (22°) as described previously[2]. The BSA concentration was always $1{\cdot}45 \times 10^{-4}$ M (1%), using a molecular

weight of 69000. The drug concentrations ranged from 4×10^{-5} M to 2×10^{-4} M. All solutions were made with 1/15 M sodium phosphate buffer and adjusted to the desired pH-value with 1 M HCl or 1 M NaOH.

Calculations. The extent of the binding of the benzodiazepines at various concentrations (c) to BSA was given by the percentage of free (α) and bound (β) drug.

The apparent binding constants k^+ and the regression coefficients or the slope constants m are obtained from the relationship[15]

$$\log c_b = \log k^+ + m \log c_f$$

where c_b and c_f are the concentrations of the bound and the free drug. k^+ can be obtained from $c_b = 1[10^{-5}$ M], if the equation is reduced to $\log c_b = \log k^+$ [15].

The number of binding sites n and the total binding constants K_1 are obtained from the reciprocal plot[16] following the equation

$$\frac{1}{\bar{r}} = \frac{1}{nk} \cdot \frac{1}{c_f} + \frac{1}{n}$$

where $\bar{r}$ = the number of moles of the benzodiazepines bound per mole BSA, c_f = the concentration of the free drug, k = the association constant, and $nk = K_1$ = the total binding constant.

From the total binding constant the free binding energy ΔF was calculated from the relationship[17]

$$\Delta F = -RT \ln K_1$$

where R = the gas law constant and T = the absolute temperature.

RESULTS

All investigated benzodiazepines are bound to BSA at pH 7·40 to a very different extent: see the percentage of drug bound β in Table 1, where the β-values range from 12% for flurazepam to 89% for dipotassium clorazepate.

The logarithmic plot after Scholtan[15] gives straight lines which regression coefficients or slope constants m are in most cases only a little less than one (Table 1). The apparent binding constants obtained from this plot are summarized in Table 1.

In order to determine the number of binding sites n and the total binding constants K_1 we used the reciprocal plot[16] in the case of the binding of the benzodiazepines to BSA and not the Scatchard plot[18] as used for the binding to HSA[2]. The reason for this is that by the Scatchard plot the number of binding sites can only be determined exactly if the value of n and the number of moles of the drug bound per mole albumin ($\bar{r}$) do not differ greatly[19]. This assumption is not given in our experiments, therefore we used the reciprocal plot, where the extrapolations of the straight lines to the ordinate are smaller than the extrapolations to the abscissa would be in the case of the Scatchard plot. The number of binding sites n and the total binding constants K_1 obtained from Scatchard or reciprocal plots are nearly identical in the case of the binding of the benzodiazepines to HSA, as shown on Table 2.

The benzodiazepines have more than one binding site on the BSA molecule, in most cases two or three (Table 1). The affinities to BSA of each derivative differ very much, which can be seen by the total binding constants K_1 (Table 1). The reciprocal plot, which is derived from mass law considerations like the Scatchard plot[17], results in straight lines (Table 1), suggesting that the binding sites (Table 1) found for the interaction with BSA are independent and equivalent[17]. The free binding energies ΔF, calculated from the total binding constants K_1 are summarized in Table 1.

A plot of the concentrations of the drug bound versus the concentrations of the free drug (Fig. 1) gives straight lines.

For further characterization of the binding of the benzodiazepines to BSA, the influence of the pH-value of the solution on the binding was determined (Fig. 2). Whereas the binding of oxazepam and dipotassium clorazepate is nearly unchanged by raising the pH-value from 6·60 to 8·20, the percentage of drug bound β of all other benzodiazepines investigated is increased by increasing the pH to 8·20 (Fig. 2).

Table 1. Binding constants*

Substance	k^+ ($(10^{-5}$ M$)^{1-m}$)	m	β (%)	$K_1 \times 10^{-4}$ (1/M)	n (M/M)	$-\Delta F^0$ (cal/mole)	r_1	r_2
Dipotassium clorazepate	8·97	0·686	89	9·084	2·32	6692	0·9893	0·9988
Lorazepam	5·28	0·752	81	4·105	2·90	6227	0·9882	0·9985
Oxazepam	4·59	0·704	77	3·675	2·02	6162	0·9918	0·9994
Tetrazepam	2·95	0·831	71	2·035	3·45	5815	0·9878	0·9994
Clonazepam	2·91	0·827	69	2·024	3·19	5812	0·9917	0·9997
Diazepam	1·31	0·888	53	0·823	5·56	5285	0·9803	0·9982
Nitrazepam	1·21	0·875	51	0·757	4·01	5236	0·9900	0·9967
Demoxepam	1·15	0·572	30	0·686	0·52	5178	0·9629	0·9964
Chlordiazepoxid	0·87	0·903	39	0·584	2·25	5084	0·9579	0·9905
Bromazepam	0·22	0·790	13	0·111	0·37	4110	0·7607	0·9337
Flurazepam	0·16	0·938	12	0·105	0·75	4078	0·8257	0·7940

* The apparent binding constants, k^+, the regression coefficients of slope constants, m, the percentage of benzodiazepines bound (β) at a total concentration $c = 1{\cdot}5 \times 10^{-4}$ M, the total binding constants, K_1, the number of binding sites of the benzodiazepine derivatives on the BSA molecule, n, and the free binding energy, ΔF. Calculated for BSA 1% at pH 7·40. r_1 = correlation coefficients of the logarithmic plots following Scholtan[15]. r_2 = correlation coefficients of the reciprocal plots[10]. In each case the regression lines represented 16 experiments at 8 different concentrations in the range from 4×10^{-5} M to 2×10^{-4} M.

Table 2. Comparison of Scatchard and reciprocal plot*

Substance	Scatchard plot*		Reciprocal plot	
	$K_1 \times 10^{-4}$ (1/M)	n (M/M)	$K_1 \times 10^{-4}$ (1/M)	n (M/M)
Diazepam	49·19	1·09	52·43	1·06
Dipotassium clorazepate	34·79	1·55	35·56	1·53
Chlordiazepoxid	21·81	1·28	19·76	1·43
Oxazepam	12·08	1·08	11·41	1·10
Tetrazepam	8·81	1·58	8·82	1·55
Lorazepam	3·76	1·22	3·93	1·14
Demoxepam	3·58	0·79	3·23	1·02
Bromazepam	1·61	0·69	1·66	0·65
Nitrazepam	1·50	1·45	1·62	1·25
Clonazepam	0·81	1·85	0·86	1·21
Flurazepam	—	—	0·078	0·22

* The total binding constants, K_1, and the number of binding sites on the HSA molecule, n, calculated from the Scatchard and the reciprocal plot for HSA 1%, pH 7·40.
† The data were taken from Müller and Wollert[2].

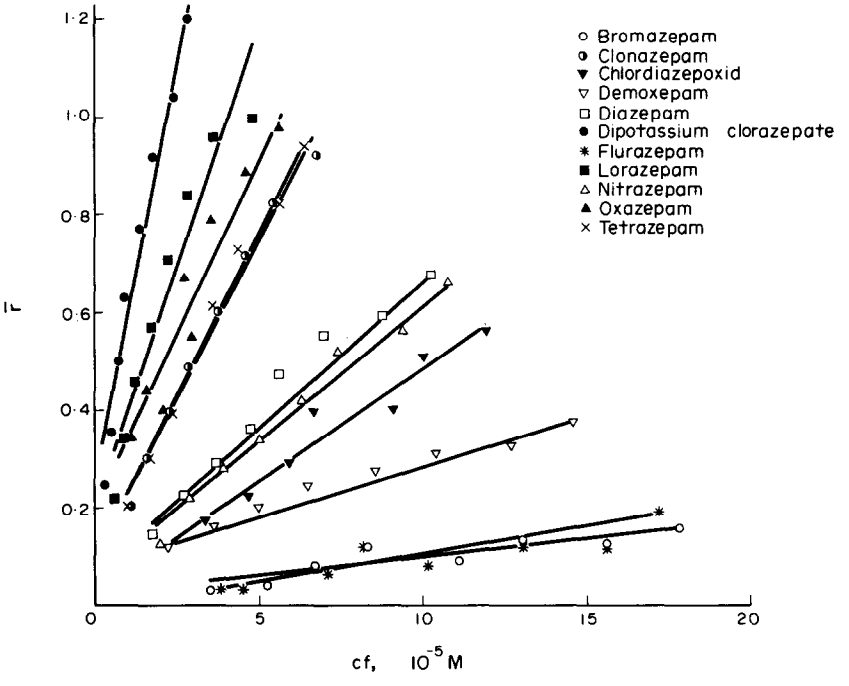


Fig. 1. Binding capacity of a 1% BSA solution for the benzodiazepines. The relationship between the number of moles of the benzodiazepines bound per mole BSA ($\bar{r}$) and the concentration of the free benzodiazepines (c_f). Ordinate: $\bar{r}$ (M/M). Abscissa: c_f (10^{-5} M). Each point represents the mean value of two experiments. The equations of the regression lines are for:

Bromazepam	$\bar{r} = 0{\cdot}025 + 0{\cdot}007\ c_f;\ r = 0{\cdot}8992$
Chlordiazepoxid	$\bar{r} = 0{\cdot}026 + 0{\cdot}046\ c_f;\ r = 0{\cdot}9790$
Clonazepam	$\bar{r} = 0{\cdot}101 + 0{\cdot}130\ c_f;\ r = 0{\cdot}9933$
Demoxepam	$\bar{r} = 0{\cdot}100 + 0{\cdot}019\ c_f;\ r = 0{\cdot}9853$
Diazepam	$\bar{r} = 0{\cdot}062 + 0{\cdot}063\ c_f;\ r = 0{\cdot}9846$
Dipotassium clorazepate	$\bar{r} = 0{\cdot}241 + 0{\cdot}358\ c_f;\ r = 0{\cdot}9792$
Flurazepam	$\bar{r} = 0{\cdot}002 + 0{\cdot}010\ c_f;\ r = 0{\cdot}8851$
Lorazepam	$\bar{r} = 0{\cdot}198 + 0{\cdot}199\ c_f;\ r = 0{\cdot}9707$
Nitrazepam	$\bar{r} = 0{\cdot}060 + 0{\cdot}056\ c_f;\ r = 0{\cdot}9888$
Oxazepam	$\bar{r} = 0{\cdot}202 + 0{\cdot}151\ c_f;\ r = 0{\cdot}9770$
Tetrazepam	$\bar{r} = 0{\cdot}094 + 0{\cdot}134\ c_f;\ r = 0{\cdot}9945$

r = correlation coefficient.

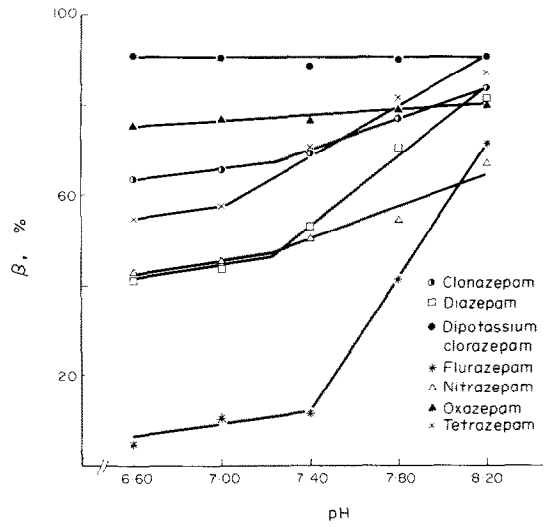


Fig. 2. Influence of pH on the percentage of benzodiazepines ($1{\cdot}5 \times 10^{-4}$ M) bound to BSA (1%). Each point represents the mean value of two experiments. Ordinate: percentage of bound drug, β. Abscissa: pH.

DISCUSSION

The intention of this study was to investigate the binding of benzodiazepine derivatives to BSA and to compare the binding to BSA with the unusual binding of these substances to HSA[2]. In regard to this, all experimental conditions chosen were identical to those in the experiments with HSA[2]. In respect to this BSA obtained from the same manufacturer and of the same quality (electrophoretic purity 100% as indicated by the manufacturer) was used. The only exception is the use of the reciprocal plot instead of the Scatchard plot. Nevertheless, the results obtained with both plots are comparable, because both plots are derived from the same binding equation[17] and both plots produce similar results in the case of the binding of the benzodiazepines to HSA (Table 2).

Before starting our investigations on the albumin binding of benzodiazepine derivatives, the binding constants for diazepam ($1{\cdot}04 \times 10^4$) and chlordiazepoxid ($1{\cdot}05 \times 10^4$), obtained for BSA 4% at 37° by ultracentrifugation experiments[20], were the only data known. Although our binding measurements are made with BSA 1% at 22°, the cited results can be satisfactorily compared with our binding constants (diazepam, $K_1 = 0{\cdot}82 \times 10^4$; chlordiazepoxid, $K_1 = 0{\cdot}58 \times 10^4$) (taken from Table 1).

The apparent binding constant k^+, obtained from the logarithmic equation after Scholtan[15] indicates more the binding capacity of the BSA molecule than its affinity for a ligand. Regarding dipotassium clorazepate, the values for k^+ are 14·5 for the binding to HSA[2] and 8·97 for the binding to BSA (Table 1) and do not differ very much. Whereas there is a great difference in the affinity constants K_1 ($K_1 = 34{\cdot}79 \times 10^4$, HSA Table 2; and $K_1 = 9{\cdot}08 \times 10^4$, BSA Table 1). The fact that the differences of the apparent binding constants between both albumins are smaller than the differences between the total binding constants can be applied to nearly all investigated benzodiazepines. This can be explained by the finding that the benzodiazepines have more binding sites on the BSA molecule than on the HSA molecule (Tables 1 and 2), which obviously must have an influence on the binding capacity of the albumins.

Another binding constant obtainable from the logarithmic equation is the slope constant m (Table 1). Nearly all m-values found for the binding of the benzodiazepines to HSA[2] are very much smaller than one, which was explained, in contradiction to Scholtan[17], by a saturation of the benzodiazepine binding site[2]. The m-values found in this study for BSA (Table 1) are in nearly all cases only slightly smaller than one, suggesting[2] that the binding sites of the benzodiazepines on the BSA molecule will not be saturated in the investigated concentration range. This suggestion is supported by a plot of the free concentrations of the ligands versus the bound concentrations (Fig. 1), which gives straight lines, and not curves as shown for HSA[2].

The large and important differences of the binding of the benzodiazepines to both albumins can be drawn from the total binding constants K_1 and the number of binding sites n (Tables 1 and 2). Nearly all benzodiazepines have more than one binding site on the BSA molecule in contrast to only one binding site on the HSA molecule and they have much smaller affinities to BSA than to HSA.

It is known that human and bovine serum albumin can differ in the binding behaviour for small organic molecules[6–11] and that often the affinity of BSA is smaller than that of HSA[7, 8, 12]. But on the other hand, up to now substances were not at all known whose affinities to both albumins differ in such degree as shown for some benzodiazepines, e.g. diazepam K_1 (HSA) = $49{\cdot}19 \times 10^4$ (Table 2) and K_1 (BSA) = $0{\cdot}82 \times 10^4$ (Table 1).

As in the case of the interaction with HSA, the hydrophobic nature of the benzodiazepines seems to have generally no greater influence on the binding of the substances to BSA, with the exception of the following examples: 2′-chlorine substitution increases the affinity to BSA (Table 1) and decreases the affinity to HSA (Table 2), which can be seen for oxazepam–lorazepam and for nitrazepam–clonazepam. In a similar way, partial hydrogenation of the B-benzene ring increases the binding to BSA and decreases the binding to HSA (see diazepam–tetrazepam, Tables 1 and 2). Both alterations of the benzodiazepine molecule increase the lipophilic character of the substances, as shown by the partition coefficients between n-octanol and 1/15 M phosphate buffer[13]. This suggests that for these substances the hydrophobic nature of the ligands is important for the binding to BSA, whereas the interaction with HSA seems to depend more on changes in the chemical structure according to the above-mentioned alterations of the substances[2]. In most cases known, changes in the chemical structure of drugs influence the binding to different albumins in a similar way[8, 11, 12]. We do not know any group of substances where relatively small changes of the chemical structure can have such opposite influences of the binding behaviour to human and bovine serum albumin as shown in this study.

Most of the hydrophobic benzodiazepines[13], namely the N_1-substituted derivatives, e.g. tetrazepam, diazepam, and chlordiazepoxid are only weakly

bound to BSA (Table 1). The affinities of these substances to BSA (Table 1) increase in the same order as the partition coefficients[13]. Possibly the N_1-substituted derivatives form a separate group within the benzodiazepines, in which the affinities increase in dependence on the hydrophobic nature of the substances.

Lorazepam and clonazepam are higher bound to BSA than to HSA (Tables 1 and 2). This is remarkable because HSA commonly has greater affinities for small ligands than BSA[7, 8, 12], only few exceptions being known[21, 22].

The binding of flurazepam is largely increased by raising the pH-value from 6·60 to 8·20 (Fig. 2), which can possibly be explained by a decrease of the cationic charged part of the molecule, as suggested for the binding to HSA[3]. In contrast to the results with HSA[3], the binding of diazepam and tetrazepam to BSA is largely increased by raising the pH (Fig. 2). Because of the pK_a-values of the benzodiazepines[3], changes of the charge of the ligands can not be the only reason for the increase of the binding of these substances. In the investigated pH-range the low alcaline structure transition of the BSA molecule takes place[23]. It seems that within this N→B transition[24] either new binding sites are available, as shown for the binding to HSA[3] or that the affinities of the binding sites, already present at pH 7·40, are increased within this structure transition.

It is very difficult to draw information from the present data on the binding part of the benzodiazepine molecule or on the binding groups of the BSA molecule, as was done for the interaction of the substances with HSA[2, 3]. For the binding to BSA the hydrophobic nature of both benzene rings of the benzodiazepine molecule seems to be relevant, which can be seen by the relative weak binding of the two 7-nitro-derivatives, nitrazepam and clonazepam, and by the increase of the binding by raising the hydrophobic nature of the B-benzene ring (tetrazepam, lorazepam, and clonazepam). The binding sites on the BSA molecule seem to be located near positively-charged groups of the protein molecule, which can be concluded (1) from the large increase in binding of flurazepam, if its cationic charge decreases (Fig. 2), (2) in part from the relatively high binding of the anionic benzodiazepines dipotassium clorazepate (Table 1) and *d*- and *l*-oxazepam hemisuccinate[5].

The increase in binding of the two nitro-derivatives (nitrazepam, clonazepam) at pH 8·20 (Fig. 2), if their anionic charges are raised[3], can be due to electrostatic forces, but also to the appearance of new binding sites, as discussed for diazepam and tetrazepam.

In summary, the unusually high differences of the binding of *d*- and *l*-oxazepam hemisuccinate to HSA and BSA[4, 5] are not mainly due to stereochemical aspects of the interaction with both albumins, but are due to generally different binding behaviours of the investigated benzodiazepines to BSA. The benzodiazepines are the first group of drugs known in which binding to human and bovine serum albumin differs qualitatively as well as quantitatively in such degree. The main reason for these large differences must be that the very specific binding site, found on the HSA molecule[2, 4] does not exist on the BSA molecule. According to the reported results, care has to be taken in future in drawing conclusions from results which are derived from experiments with BSA alone, as done by several authors[25–28]. In agreement with Witiak *et al.*[21] we think that drug binding studies can only be of pharmacokinetic significance for man if they are obtained from experiments with human serum albumin.

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