Biochemical Pharmacology, Vol. 25, pp. 2131–2140. Pergamon Press, 1976. Printed in Great Britain.

STUDIES ON THE BINDING OF BENZODIAZEPINES TO HUMAN SERUM ALBUMIN BY CIRCULAR DICHROISM MEASUREMENTS*

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(*Received* 29 *October* 1975; *accepted* 24 *March* 1976)

Abstract—The binding of twenty-one different benzodiazepine derivatives to human serum albumin (HSA) has been studied by circular dichroism (CD) measurements in 0.1 M KCl and 0.005 M phosphate buffer at pH 7.4 and 25°. The binding has been related to the qualitative changes of the CD spectra of HSA between 250 and 350 nm and, in some representative benzodiazepine derivatives, to the electron distribution as calculated by the CNDO/2-method. The binding has also been quantitatively studied with a continuous CD titration technique. The data were numerically analyzed with computer programs based on one-site, two-sites and three-sites models. It is concluded that most derivatives will bind primarily to one site on HSA. It is moreover concluded that variations of the C_2-amino side chains will not influence the binding properties. Oxygens at C_2 or C_3 will increase the binding, while oxygens in both positions will decrease the binding. A derivative with a C_7-amino group will show only weak affinity for HSA, which might be explained by the positive character of the hydrogens in the amino group according to the CNDO/2-calculation. A C_7-nitro group will also impair the binding, as well as large substituents at N_1.

Many benzodiazepine derivates are widely used in the treatment of various mental depressions and other psychic disorders and much information is available about their clinical and pharmacological effects [1]. They have been shown to bind in blood to human serum albumin (HSA) by circular dichroism (CD) and gelfiltration studies [2–4]. However, detailed qualitative and quantitative information is still lacking about the binding of the drugs and of their metabolic degradation products to HSA, which is necessary to allow conclusions to be drawn about the pharmacokinetic significance of the binding. Such information is also necessary to gain knowledge about the structural and conformational requirements for strong binding.

Müller and Wollert [4, 5] have shown by gel filtration that benzodiazepine derivatives are mainly bound to one site on the HSA molecule, and that the degree of binding increases with pH. The increased binding is in some cases due to an increased association constant and in other cases to an increased number of sites. They have also shown that the binding of oxazepam hemisuccinate to HSA and bovine serum albumin is stereospecific [6, 7].

Several different benzodiazepine derivatives are available enabling the relation between the structure of the derivatives and their affinity to HSA to be studied. For this purpose we have applied CD measurements, which are very informative, as the Cotton effects induced when chromophores are interacting with an asymmetric surface can be used to identify the chromophore and also can be related to the orientation of the chromophore in the complex [8]. Moreover, by continuously adding the drugs to HSA and numerically analyzing the increase of the Cotton effects according to the method described in the Appendix (following paper), the binding constants can be determined. The results have also been related to the electron distribution in some drugs, estimated by the CNDO/2-method.

MATERIALS AND METHODS

Human serum albumin (HSA) was prepared from outdated blood and purified according to McMenamy *et al.* [9] and Chen [10], essentially as described earlier [2]. The concentration of HSA was determined from the optical density at 280 nm ($A^{1\%}_{1\,cm} = 5.8$, [11]). The molecular weight, 66,300, was estimated from the primary structure as presented by Behrens *et al.* [12].

Benzodiazepine derivatives were obtained as gifts from Roche-Produkter AB, Stockholm except oxazepam and triazolam, which were obtained, respectively, from KABI AB, Stockholm, and from The Upjohn Comp., Kalamazoo, Michigan, U.S.A. The chemical structures and the names of the compounds are shown in Table 1. The drugs were used without further purification.

Circular dichroism (CD) was studied with an automatic spectropolarimeter, JASCO J-20, Japan Spectroscopic Comp., Tokyo, at 25° in thermostated cell-holders. The instrument was calibrated with D-10-camphorsulfonic acid [13] and checked before every experiment. The circular dichroism is expressed as molar ellipticity, $[\theta]$, in degrees $cm^2\,dmole^{-1}$, calculated with reference to the HSA concentration or as difference molar ellipticity, $\Delta[\theta]$. All measurements were made in 0.1 M KCl and 0.005 M sodium phosphate buffer, pH 7.4.

* This paper is no 4 in a series entitled "Binding of Drugs to Human Serum Albumin". For no 3, see *Biochem. Pharmac* **25**, 1205 (1976).

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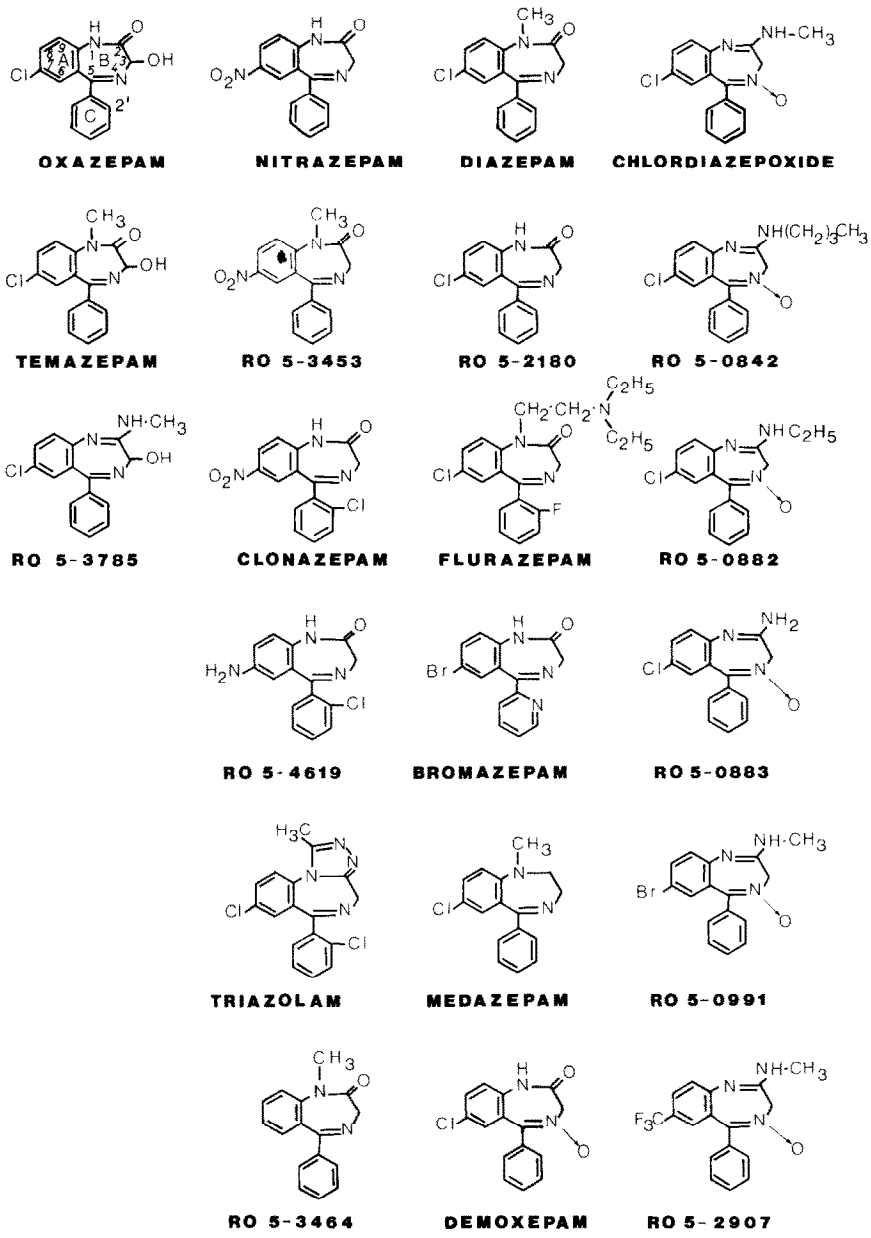


Table 1. Chemical structures of the benzodiazepine derivatives

Absorption spectra were taken with an automatic double-beam spectrophotometer, Shimadzu MPS-5000. The absorbance of the drugs was measured in 0.1 M KCl and 0.005 M sodium phosphate buffer, pH 7.4.

Spectrophotometric determination of pK_a. The dissociation constants of compounds for which values could not be found in the literature were determined spectrophotometrically with the Shimadzu MPS-5000 spectrophotometer. The pH was measured with a Radiometer PHM 26 pH-meter with a 10× scale expander. The substances were dissolved in 0.1 M KCl and difference spectra were recorded between 240 and 330 nm. The pH in the reference cell was 1.0–1.5 and was changed in the sample cell up to about pH 12. The changes of the absorption at selected wavelengths were plotted against pH and the pK_a-values were then determined graphically.

Preparation of solutions. A stock solution of HSA in 0.005 M sodium phosphate with 0.1 M KCl, pH 7.40 ± 0.04, was prepared. To a fixed volume of this solution was added either (A) the drug under study, dissolved in ethanol to a drug–protein ratio of about 15 or 1 or (B) buffer, so that the HSA concentrations was the same as in (A). The total ethanol concentration did not exceed 0.4%, except in the case of medazepam, when an ethanol concentration of maximally 0.7% was reached.

Determination of the binding constants was done by the CD titration described in the Appendix. The drug–protein solution (A) was continuously added to solution (B) at 25° until approximately a 5- to 10-fold

Table 2. Association constants for the binding of benzodiazepines to human serum albumin and their protolytic dissociation constants

Compound	Binding constants: One-site program $K \times 10^{-4}$ (M^{-1})	Two-site program $K_1 \times 10^{-4}$ (M^{-1})	Two-site program $K_2 \times 10^{-4}$ (M^{-1})	Protolytic dissociation constants pK_{a1}*	pK_{a2}
Ro 5-3785	61	‡	‡	6.0	
Ro 5-2180	19	73	lower limit	3†	
Diazepam	18	39	lower limit	3.3 [21]	
Ro 5-0842	14	11	lower limit	4.3	
Ro 5-0882	11	6.0	lower limit	4.3	
Ro 5-0991	10.4	15	lower limit	4.8	
Ro 5-3464	8.7	52	1.2	3.3†	
Medazepam	8.5	2.8	lower limit	4.4 [21]	
Ro 5-2907	5.5	49	lower limit	4.3	
Oxazepam	4.3	9.6	lower limit	1.7 [21]	11.6 [21]
Temazepam	4.2	2.6	0.59	< 2	
Chlordiazepoxide	3.9	25	1.3	4.9	
Ro 5-4619	n.d.			< 3	
Ro 5-0883	n.d.			4.9	
Bromazepam	n.d.			2.8 [5]	11.7 [5]
Clonazepam	n.d.			1.5 [22]	10.5 [22]
Demoxepam	n.d.			1.5 [5]	12.0 [5]
Flurazepam	n.d.			1.4–1.6 [5, 23]	8.4 [5]
Nitrazepam	n.d.			2.9–3.2 [5, 21]	10.8 [21]
Triazolam	n.d.				
Ro 5-3453	n.d.				

The binding constants have been obtained by numerical analyses of data from continuous circular dichroism titration in 0.1 M KCl and 0.005 M phosphate buffer at pH 7.4 and 25°. Two different programs based on one-site and two-site models have been used for the numerical calculation. The values are means from two or three analyses. The protolytic dissociation constants have been determined spectrophotometrically at 25°, when not taken from the literature.

* Corresponds to the dissociation constants of the protonated bases, HB^+.

† Estimated values.

‡ Implausible values obtained.

molar excess of drug over HSA was attained. From batch experiments, it was noted that the equilibrium between HSA and the drug was reached rapidly. The ellipiticity of the solution in the mixing vessel was continuously measured at a fixed wavelength chosen to give the best measuring conditions for each drug. Generally, the ellipticity was recorded at 262 nm, where the CD spectrum of HSA shows a minimum. The concentrations of HSA and the drugs were chosen to get a reasonable excess of ligand and a measurable change in ellipticity.

The CD curve obtained with increasing amounts of drugs was analyzed numerically as described in the Appendix.

Quantum mechanical calculations. The electron density was calculated by the CNDO/2 method (complete neglect of differential overlap) [14]. The computer program used was restricted to 80 atomic orbitals and therefore some simplifications were necessary. The bond distances and bond angles for the different atoms have been taken from Sutton's Tables [15] or else estimated from analogous compounds. The diazepine ring was assumed to be completely planar with the C—C, N—C and C—H distances measuring 0.1395 nm, 0.1395 nm and 0.1095 nm, respectively. The angles in the six-membered ring were all taken to be 120° and the angles in the diazepine ring to be 128.57°.

Equilibrium dialysis was carried out in 1 ml dialysis cells at room temperature with three derivatives, two cells being used for each drug. In one of the cells the drug was dialysed against the buffer (0.1 M KCl with 0.005 M sodium phosphate, pH 7.4), and in the other against an albumin solution, 0.1×10^{-3} M in buffer. The molar relation of triazolam, Ro 5-3453 and Ro 5-4619 was 0.4, 0.9 and 0.8 times that of HSA, respectively. 1.0 ml of the solutions were pipetted into the dialysis cells. Technicon Type A standard membranes were used in the cells. The time used for equilibration was 8 hr. The drug remaining on the buffer side was determined spectrophotometrically.

RESULTS

Determination of the pK_a-values. The pK_a-values of the drugs studied, obtained from the literature and from our own spectrophotometric determinations, are summarized in Table 2. As can be seen, all derivatives except flurazepam and Ro 5-3785 are uncharged at pH 7.4. Flurazepam has an N_1-side chain, which is largely positively charged. Ro 5-3785 is protonated at pH 6.0 according to spectrophotometric titration, and in paper electrophoresis it is stationary at pH 8.0 and at pH 5.0 it migrates towards the cathode. The pK_{a_1} value of 6.0 means that about 4 per cent of the substance will be protonated at pH 7.4, which might complicate the binding studies.

The N_4-atom is protonated at low pH-values, generally around pH 3.3 and lower (compare diazepam, nitrazepam and oxazepam). The benzodiazepox-

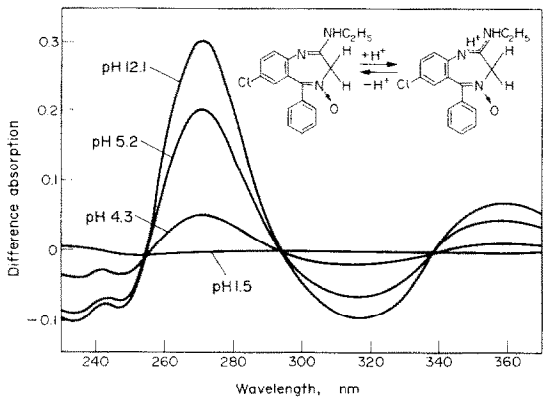


Fig. 1. Spectrophotometric titration of Ro 5-0882. Difference absorption spectra are shown of Ro 5-0882 at pH 4.3, 5.2, 12.1 with the same solution at pH 1.5 in the reference cell. The concentration was 0.32×10^{-4} M.

ides have pK_a-values around 4.5, which can be ascribed to the formation of an amidinium structure, as shown by Möhrle *et al.* in NMR studies [16]. In Fig. 1, it is shown that the formation of the amidinium structure is accompanied by a blue shift of a 358 nm band to 317 nm and of a 271 nm band to 247 nm. Similar changes are observed when Ro 5-3785 is protonated at pH 6.0, which confirms that N_1 and the amino side chain are the proton-accepting groups.

Qualitative binding studies. Twenty of the twenty-one benzodiazepine derivatives investigated form complexes with HSA in such a way that the CD spectrum of HSA is substantially changed between 250 and 350 nm, the only exception being Ro 5-4619. Representative examples are shown in Fig. 2, where the CD spectra of HSA and of HSA with appropriate excess of diazepam, nitrazepam, oxazepam and chlordiazepoxide can be seen. However, the characteristics

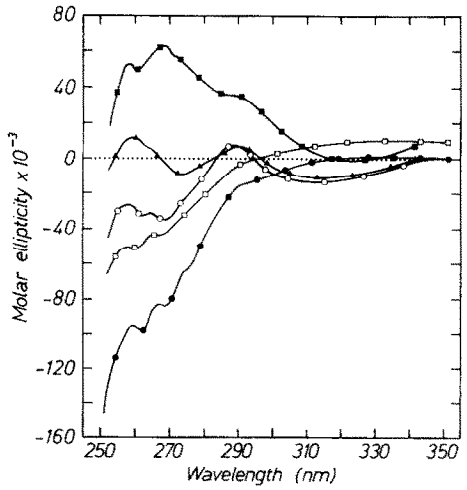


Fig. 2. CD spectra of HSA (—●—) and of HSA with the following drugs: chlordiazepoxide (4.6-fold molar excess over HSA, ···■···), diazepam (4.0-fold molar excess, —▲—), oxazepam (3.8-fold molar excess, —○—), nitrazepam (3.8-fold molar excess, —□—). The protein concentrations were 0.24×10^{-4}, 0.11×10^{-4}, 0.24×10^{-4}, 0.22×10^{-4} and 0.22×10^{-4} M, respectively.

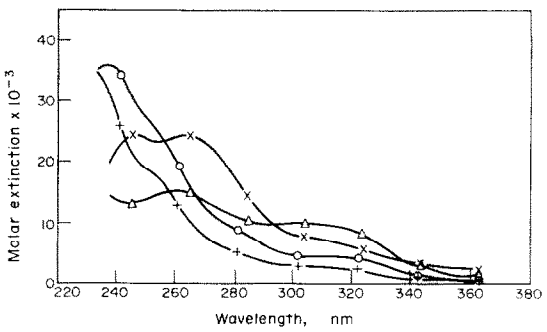


Fig. 3. Absorption spectra of diazepam (··· + ···), nitrazepam (—△—), oxazepam (—○···) and chlordiazepoxide (··· × ···) in ethanol-water, at pH 7.4.

of the induced Cotton effects can be studied more conveniently in the difference CD spectra obtained after subtraction of the contribution from HSA itself, shown in Figs. 4–7. Thereby we have used a drug concentration, which was high enough to attain a reasonably large saturation degree of the HSA binding site. (Generally a 4-fold molar excess of drug over HSA was chosen). The CD difference spectra show some similarities with the absorption spectra, as seen in Fig. 3.

Below about 300 nm and as far as the ellipticity was recorded, the induced Cotton effects will add positive ellipticity to that of HSA. Beyond that wavelength, negative Cotton effects are induced when complexes of HSA with Ro 5-2180 (Figs. 4, 7), Ro 5-3464 (Fig. 4), diazepam (Figs. 4, 6), temazepam (Fig. 6), oxazepam and Ro 5-3785 (Fig. 7) are formed. It is important to emphasize in this context that the benzodiazepines studied were mixtures of different steric forms and thus will not exhibit any ellipticity of their own.

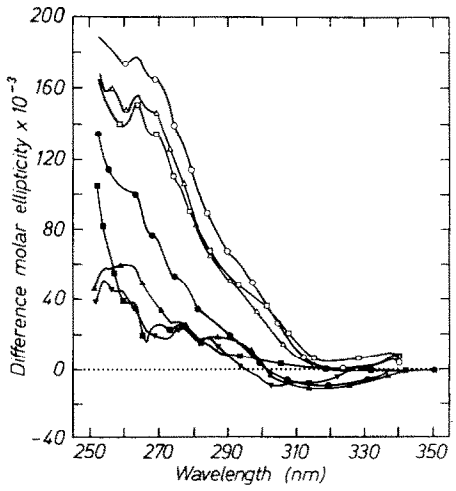


Fig. 4. Difference CD spectra of HSA with the following drugs: Ro 5-0991 (5.0-fold molar excess over HSA, ···○···), chlordiazepoxide (4.6-fold molar excess, ···△···), Ro 5-2907 (4.8-fold molar excess, ···□···), diazepam (4.0-fold molar excess, —●—), Ro 5-2180 (2.5-fold molar excess, ···▲···), demoxepam (3.7-fold molar excess, ···■···), Ro 5-3464 (3.4-fold molar excess, ···▼···). The HSA concentrations were 0.22×10^{-4}, 0.11×10^{-4}, 0.11×10^{-4}, 0.24×10^{-4}, 0.77×10^{-5}, 0.24×10^{-4} and 0.73×10^{-4} M, respectively.

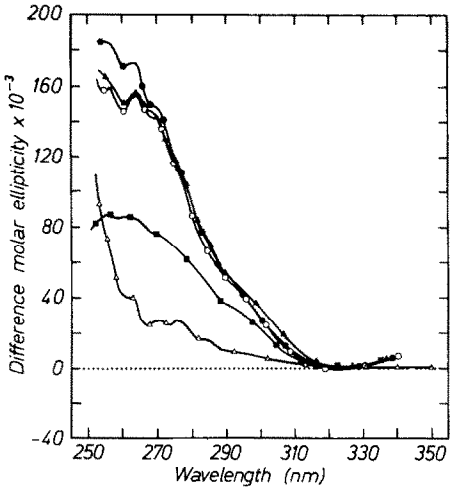


Fig. 5. Difference CD spectra of HSA with the following drugs: Ro 5-0842 (4.6-fold molar excess over HSA, —●—), Ro 5-0882 (4.9-fold molar excess, —▲—), chlordiazepoxide (4.6-fold molar excess, —○—), Ro 5-0883 (5.3-fold molar excess, —■—), demoxepam (3.7-fold molar excess, —△—). The protein concentrations were 0.11×10^{-4}, 0.11×10^{-4}, 0.11×10^{-4}, 0.12×10^{-4} and 0.24×10^{-4} M, respectively.

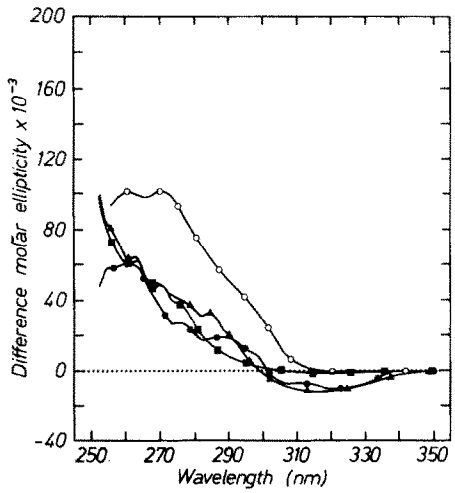


Fig. 7. Difference CD spectra of HSA with the following drugs: Ro 5-3785 (4.5-fold molar excess over HSA, —○—), oxazepam (3.8-fold molar excess, —▲—), bromazepam (4.0-fold molar excess, —■—), Ro 5-2180 (2.5-fold molar excess, —●—). The protein concentrations were 0.15×10^{-4}, 0.22×10^{-4}, 0.22×10^{-4} and 0.77×10^{-5} M, respectively.

The difference CD spectra of the drug-HSA complexes are arranged in Figs. 4–7 as far as possible in order of their structural similarities, i.e. benzodiazepine oxides, nitrated benzodiazepines and hydroxylated benzodiazepines. However, to allow an adequate comparison of the effect on the spectra of different substituents, some spectra are shown in more than one figure.

Figure 4 shows that a change of the C_7 substituent in the A-ring of the benzodiazepine oxides from Cl to Br or CF_3 (chlordiazepoxide, Ro 5-0991, Ro 5-2907) has no effect on the difference CD spectra. Also, the change in the difference CD spectra by altering the substituent at N_1 (diazepam vs Ro 5-2180) and N_4 to an oxide (demoxepam vs Ro 5-2180) can be compared. In the former case, the methyl group at N_1 will enhance the positive Cotton effect at 262 nm. (Even if the excess of Ro 5-2180 is only 2.5-fold, the binding site is almost saturated at the concentrations used.) With the oxide (demoxepam) the negative ellipticity at 310–320 nm is lost, while the spectrum at 255–300 nm is mainly unchanged. In addition, the importance of the chlorine at C_7 for a strong positive Cotton effect (diazepam vs Ro 5-3464) should be noted.

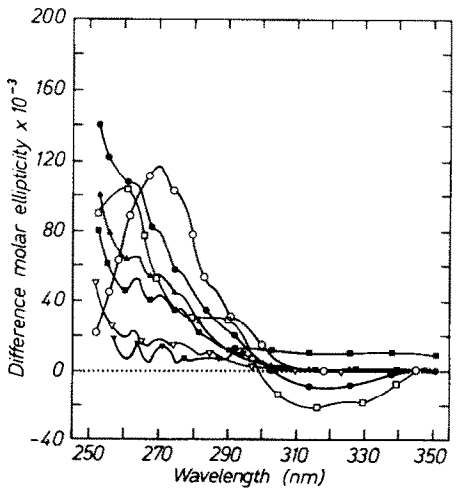
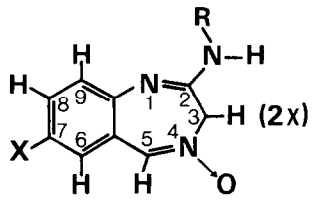


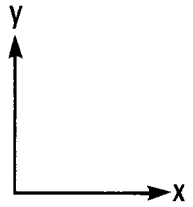
Fig. 6. Difference CD spectra of HSA with the following drugs: medazepam (9.0-fold molar excess over HSA, —○—), diazepam (4.0-fold molar excess, —●—), temazepam (9.0-fold molar excess, —□—), clonazepam (4.0-fold molar excess, —▲—), nitrazepam (3.8-fold molar excess, —■—), flurazepam (2.5-fold molar excess, —▽—), Ro 5-3453 (7.7-fold molar excess, —▼—). The protein concentrations were 0.78×10^{-5}, 0.24×10^{-4}, 0.78×10^{-5}, 0.22×10^{-4}, 0.22×10^{-4}, 0.22×10^{-4} and 0.78×10^{-5} M, respectively.

In Fig. 5, the consequences of a change of the B-ring structure can be noted, when comparing chlordiazepoxide, demoxepam and Ro 5-0883. Demoxepam gives rise to a significantly smaller Cotton effect, from which it can be concluded that the strong positive Cotton effect at 270–280 nm can be attributed to the influence of the —N=C< group. (Compare also Fig. 7, where the spectra with Ro 5-3785 and oxazepam are shown and Fig. 1, where the result of the spectrophotometric titration of Ro 5-0882 is shown.) In Fig. 5 is also shown that different amino side chains at C_2 are of minor importance for the difference spectra (compare chlordiazepoxide, Ro 5-0842, Ro 5-0883 and Ro 5-0882).

In Fig. 6, three comparisons can be made; the influence of the C_7 substituent (diazepam vs Ro 5-3453) the influence of the N_1 substituent (Ro 5-3453 vs nitrazepam) and the influence of a chloro-substituted C-ring (clonazepam vs nitrazepam). A common feature of the C_7-nitro substituted benzodiazepine derivatives is that small Cotton effects arise when they are bound to HSA. The methyl group on N_1 in diazepam has a positive effect on the CD spectrum (Fig. 4). The same effect is seen when temazepam is com-

Table 3. Net electron densities on benzodiazepine-oxide structures, as calculated with the CNDO/2 method





		NET ELECTRON DENSITY ON:									
X	R	$C^{(N_1-C_9)}$	C_2	C_6	C_7	C_8	C_9	$C^{(C_5-C_6)}$	$H^{(C_6)}$	$H^{(C_8)}$	$N^{(C_2)}$
$-CF_3$	$-H$	+0.132	+0.274	+0.027	-0.075	+0.038	-0.037	-0.014	+0.008	+0.002	-0.206
$-F$	$-CH_3$	+0.102	+0.255	-0.045	+0.208	-0.041	-0.015	+0.008	+0.013	+0.011	-0.156
$-Cl$	$-CH_3$	+0.112	+0.256	-0.006	+0.052	-0.002	-0.026	-0.005	-0.002	-0.004	-0.156
$-Cl$	$-H$	+0.113	+0.265	-0.005	+0.052	-0.002	-0.027	-0.005	-0.002	-0.004	-0.208

$-CF_3$	$-H$	C: +0.628	F: -0.232	F: -0.232	F: -0.229	H: +0.141
$-F$	$-CH_3$	F: -0.210	C: +0.092	H: +0.009	H: +0.009	H: +0.003
$-Cl$	$-CH_3$	Cl: -0.073	C: +0.092	H: +0.009	H: +0.009	H: +0.003
$-Cl$	$-H$	Cl: -0.072	H: +0.137			

		$DIPOLE\ MOMENT_X$	$DIPOLE\ MOMENT_Y$	$DIPOLE\ MOMENT_{TOT}$
$-CF_3$	$-H$	4.227 D	3.854 D	5.720 D
$-F$	$-CH_3$	3.554 D	3.720 D	5.145 D
$-Cl$	$-CH_3$	3.505 D	3.846 D	5.204 D
$-Cl$	$-H$	3.403 D	3.870 D	5.153 D

pared with oxazepam (Figs. 6 and 7). On the other hand, the negative effect of the methyl group in Ro 5-3453 should be noted. A chloro-substituted C-ring influences the CD spectrum of a complex only to a small extent, as seen with clonazepam.

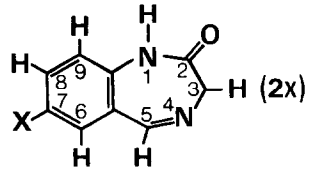
In Fig. 6, one can also see the effect of a ketone group on C_2 and/or a hydroxy-group on C_3 (medazepam vs diazepam vs temazepam) on the difference CD spectra of benzodiazepine-HSA complexes. The increasing negative band above 300 nm with increasing number of oxygen atoms at C_2 C_3 should be observed. Correspondingly, when the difference CD-spectra of the 1:1-complexes with Ro 5-2180 and oxazepam were measured, oxazepam gave larger negative ellipticity around 320 nm, despite the fact that the binding constant is 4.4 times smaller than that of Ro 5-2180. In addition, the weak difference spectrum of flurazepam is also shown.

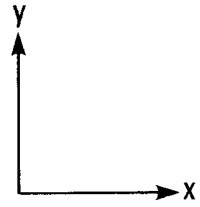
In Fig. 7, the very strong spectrum of Ro 5-3785 is to be observed. The maximum of the spectrum is, in comparison with all the other spectra, significantly shifted towards a higher wavelength, the underlying reasons for this change being unknown. From the difference CD spectrum obtained with bromazepam it can be noted that the negative ellipticity around 320 nm is comparatively small.

Quantitative binding studies. Table 2 compiles the found association constants for the binding of the benzodiazepine derivates to HSA. Ro 5-4619, triazolam and Ro 5-3453 gave no or very small Cotton effect with HSA. To check if they nevertheless were bound without changing the ellipticity, their possible binding to HSA was investigated by equilibrium dialysis. The binding degree of the three compounds to HSA was 4%, 4% and 16%, respectively. Thus, the binding affinity for HSA is indeed insignificant. The results indicate that the theoretical binding constants will not exceed $2 \times 10^3\ M^{-1}$.

Quantum mechanical calculations. The results of the calculations of the electron density are shown in Tables 3 and 4. The accuracy of the net electron density is considered to be ± 0.005. Unfortunately, only

Table 4. Net electron densities on benzodiazepine-2-one structures, as calculated with the CNDO/2 method





	NET ELECTRON DENSITY ON:						
-X	C_2	$C^{(C_5-C_6)}$	C_6	C_7	C_8	C_9	$C^{(C_9-N_1)}$
$-NO_2$	+0.337	-0.031	+0.049	-0.010	+0.048	-0.059	-0.034
$-NH_2$	+0.331	-0.022	-0.024	+0.119	-0.013	-0.034	-0.059

-X	N_1	$H^{(N_1)}$	$O^{(C_2)}$	$H^{(C_5)}$	$H^{(C_6)}$	$H^{(C_8)}$	$H^{(C_9)}$
$-NO_2$	-0.198	+0.138	-0.354	-0.004	+0.029	+0.029	+0.012
$-NH_2$	-0.206	+0.132	-0.363	-0.011	+0.001	0	0

$-NO_2$	N: +0.494	O: -0.346	O: -0.345
$-NH_2$	N: -0.262	H: +0.105	H: +0.105

-X	$DIPOLE\ MOMENT_X$	$DIPOLE\ MOMENT_Y$	$DIPOLE\ MOMENT_{TOT}$
$-NO_2$	1.3310 D	1.5817 D	2.0672 D
$-NH_2$	-4.3632 D	-1.4736 D	4.6053 D

eighty orbitals could be treated in the computer program and therefore some simplifications had to be made. Space-filling models indicate that the C-ring is probably not situated in the same plane as the 7-membered diazepine ring owing to steric hinderance. Thus, its π-electron system can be conjugated only to a minor extent with that of the rest of the molecule. The influence from the phenyl ring on the electron distribution was therefore assumed to be constant, regardless of the chemical modifications in the C-ring. A hydrogen atom has consequently been substituted for the ring in the calculations. Moreover, NMR-studies have shown that the hydrogens at C_3 are not equivalent [17–19]. This indicates that the B-ring is not planar, which also X-ray diffraction studies have shown [20]. As precise data about the conformation at 25° for the different derivatives are lacking, we used a planar B-ring in our calculations. This means that the π-electrons will be somewhat more delocalized than they really are. As the effect of a bromo-substituent (Ro 5-0991) could not be calculated, the analogous fluoro- and chloro-derivates are compared. Owing to the restrictions imposed on the calculations the values obtained should be regarded as relative net electron densities in the group studied.

DISCUSSION

All of the twenty-one benzodiazepines studied—with one exception, Ro 5-4619—interact with HSA in such a way that the CD spectrum of HSA is changed. Of substances known to bind to HSA, only acetylsalicylic acid has earlier been shown not to change the CD spectrum [2]. In the present study, it has been unequivocally shown by equilibrium dialysis that the binding of Ro 5-4619 to HSA, as well as of triazolam and Ro 5-3453 giving rise to only small CD changes, indeed is very low ($K < 2 \times 10^3\ M^{-1}$). Other benzodiazepines, however, bind strongly to HSA, giving large changes of the CD spectrum of HSA, from which it can be concluded that the difference spectra obtained with the different substances can form a basis for discussion about their qualitative and semiquantitative binding.

The observed difference CD spectra can arise from intrinsic and/or extrinsic Cotton effects. The intrinsic Cotton effects might be due to conformational changes around the chromophoric side-chains of HSA (tryptophan, tyrosine, phenylalanine and cystine) or might arise from the benzodiazepines, as these are asymmetric molecules. NMR studies of several benzodiazepines have shown that the hydrogens at C_3 are not equivalent, implying that their B-rings are not

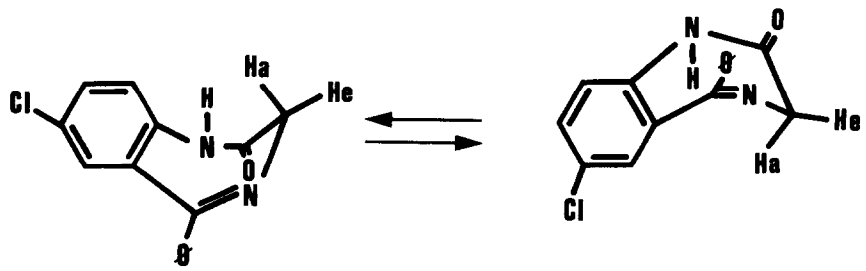


Fig. 8. Conformation of the benzodiazepine molecule, mainly according to Bley *et al.* [18].

planar, but most probably have the boat conformation [17 19]. This conclusion is supported by X-ray studies [20]. As can be seen in Fig. 8, the molecules lack an S_1-axis and have two configurations, which are optical enantiomers. However, the NMR studies also indicated that the two isomers are in equilibrium with each other also below the coalescence temperature and that the lifetime of the respective configurations, A and B, is much less than 1 sec. Thus, e.g. the lifetime at 23° of the conformations of chlordiazepoxide having the coalescence temperature 41° is estimated to 0.019 sec. [18]. Since they are energetically equal, A and B will be present in equal amounts before binding. Suppose the binding site on HSA is absolutely stereospecific and only bind conformation B. In such a case, with for example a binding degree of 70 per cent, 85 per cent of the total concentration—including the bound fraction—will have conformation B and 15 per cent conformation A. The mixture will not be racemic and will show intrinsic Cotton effect. An asymmetric C_3 (as in oxazepam, temazepam and Ro 5-3785) will not change the situation in principle. There will be four asymmetric conformations, which in pairs are in equilibrium forming a racemic mixture. The stereospecific binding site on HSA will change the equilibrium and induce optical activity in the mixture of benzodiazepine conformations. The magnitude of the induced intrinsic optical activity depends mainly on the sterospecificity of the interaction between the substance and the HSA-binding site.

The CD difference spectra and absorption spectra obtained with the benzodiazepines and HSA have several general characteristics in common. This means that the contributions from any changed intrinsic ellipticity of HSA itself must be small and can be disregarded. The induced *intrinsic* Cotton effects of the benzodiazepines themselves and the induced *extrinsic* Cotton effects, produced by perturbation of the chromophores when they are bound to the HSA-surface, will be centred at the same wavelengths, coinciding with those transitions forming the absorbtion spectra. It is therefore difficult to estimate the relative importance of the induced *intrinsic* Cotton effects for the magnitude of the CD difference spectra. Generally, the induced ellipticity is very high, $\Delta\theta > 10^5$ degrees cm^2 $dmole^{-1}$ around 260 nm when the benzodiazepines are bound. The intrinsic optical activity of the isolated enantiomers of oxazepam hemisuccinate is smaller, about 4×10^4 degrees cm^2 $dmole^{-1}$ around 260 nm [6]. It is reasonable to assume that the *induced* intrinsic effects, if present, will not significantly exceed this value. Under such circumstances, it can be concluded that the CD changes seen, when benzodiazepines bind to HSA, mainly originate from extrinsic Cotton effects.

From the CD difference spectra it should be possible to identify the chromophores involved in the binding. However, the great number of transitions forming each spectrum and the unpredictable influence on the electronic transitions from the different substituents and the protein make a conclusive identification of the different individual transitions difficult. The strongest bands should originate from the $\pi \rightarrow \pi^*$-transitions of the substituted A- and C-rings. The $n \rightarrow \pi^*$ transitions from the N and O atoms should be weak and masked by the stronger $\pi \rightarrow \pi^*$-transitions, at least below 300 nm.

In Fig. 1, it is possible to identify a strong absorption band originating from the A-ring. During the protonation, a mesomericly stabilized amidinium system is formed involving the $-\overset{1}{N}=\overset{2}{C}<$ structure, as found by NMR measurements [17]. The strong band at 271 nm is concomitantly lost and shifted to 247 nm due to the considerably decreased electron-donating properties of the amidinium structure. Similar shifts were seen with Ro 5-3785 at pH 6.0, while e.g. temazepam did not show the same spectral changes upon protonation. The band around 270 nm is present in the spectra of all derivatives with an amino side-chain at C_2. The corresponding CD band is also relatively strong, indicating that the chromophore is situated close to an asymmetric centre at the HSA binding site.

A characteristic feature of several difference CD spectra is the negative band around 315 nm. It is missing in the spectra obtained with substances having an amino group at C_2 and with the nitro derivatives. It is weak or very weak with demoxepam and bromazepam as found also by Müller and Wollert [3], but not detectable with medazepam lacking the keto group at C_2. The findings indicate, that the 315-nm band thus might originate from the $n \rightarrow \pi^*$-transition of the carbonyl oxygen at C_2. A $\pi \rightarrow \pi^*$-transition from the C-ring can be excluded, as tetrazepam (with a cyclohexane as C-ring) also gives a strong negative Cotton effect at 310 nm.

The CD difference spectra shown (Figs. 4–7) are obtained with about 4-fold molar excess of the benzodiazepines. This means that with some compounds the binding site on HSA is almost saturated, while others will only partly saturate the site correspondingly to their binding constants. The magnitude of the Cotton effects must be related to the saturation degree and to the way the chromophores are arranged in the complex. The more rigidly and tightly they are bound, the larger the Cotton effects will be [8].

Based on these principles some tentative conclusions can be drawn about the influence of different substituents on the binding.

Substituents at N_1. Large substituents at N_1 will seriously impair the affinity for HSA by steric hindrance. This fact can be exemplified with flurazepam (Fig. 6) and triazolam which show insignificant induced Cotton effects with HSA. The same has earlier been seen also with prazepam having a methylcyclopropane substituent [3].

On the other hand a methyl group will increase the Cotton effects as can be concluded when comparing diazepam with Ro 5-2180 (Fig. 4) or temazepam with oxazepam (Figs. 6 and 7). However, the consequences for the magnitude of the binding constants are negligible.

Substituents at C_2 and C_3. The bulk of the amino side chains seem to be unessential both for the size of the Cotton effects and for the binding constants (Fig. 5 and Table 2). The electron density is also similar in all the derivatives (Table 3). All the compounds with amino groups at C_2 generally give rise to strong Cotton effects centered at about 270 nm as discussed earlier.

The carbonyl oxygen at C_2 will increase the binding affinity (compare diazepam–medazepam) and a hydroxyl group at C_3 (Ro 5-3785) will give a derivative which has the highest binding constant of all the benzodiazepines studied. However, the combination of two electronegative oxygens at C_2 and C_3 will weaken the affinity to HSA. This effect is obvious when temazepam is compared with diazepam and oxazepam with Ro 5-2180. In both cases there is a 4- to 5-fold decrease of the binding constants.

N_4-oxides. A similar effect as described above is noted with the benzodiazepine oxides. A second oxygen at C_2 (demoxepam) will greatly impair the binding to HSA. The Cotton effects induced are indeed so small and appear at so high concentrations of the drug, that a meaningful numerical analysis to calculate the binding constant could not be made. N_4-oxides will not show any negative ellipticity beyond 300 nm.

Substituents at C_7. The influence on the binding of six different C_7-substituents was studied in the present work, namely H, Cl, Br, CF_3, NH_2 and NO_2. As can be seen from the electron distribution (Table 3 and 4), the mentioned substituents are negative, with the exception of the NH_2 group, in which the hydrogens are positive. This amino group is present in Ro 5-4619, which is the only compound giving no Cotton effects with HSA and insignificant binding to HSA. The amino group is the characteristic feature of Ro 5-4619, which otherwise does not contain any group preventing the binding. This implies that a positive charge around C_7 is incompatible with binding.

A chloro atom at C_7 (e.g. diazepam compared with Ro 5-3464, Fig. 4) will improve the binding, while a bromo or trifluoromethyl group (see the benzodiazepine oxides, Fig. 4) will not further change the binding properties. These conclusions are also supported by the binding constants presented in Table 2.

The derivatives containing a C_7-nitro group will generally generate small Cotton effects with HSA. Unfortunately, this means that difficulties will arise when the binding constants are to be calculated, as discussed later. However, nitrazepam and clonazepam have earlier in gelfiltration studies by Müller and Wollert been shown to give relatively small binding constants [4]. It can thus be concluded that the nitro group will impair the binding to HSA.

Substituents at C_2. The possibilities to draw definite conclusions about the effect of introducing substituents in the C-ring are small with the twenty-one substances under study. Some of the derivatives with C_2-substituents show no or very small binding tendency due to negative effects of other groups (Ro 5-4619, triazolam and flurazepam). The influence of a chlorosubstituent can be seen when comparing clonazepam and nitrazepam, the former giving somewhat larger Cotton effects below 290 nm. No quantitative evaluation has, however, been possible, but gelfiltration indicates that the primary binding constant is smaller with clonazepam than with nitrazepam [4].

Replacing the C-phenyl ring (Ro 5-2180) with a pyridine ring (bromazepam, Fig. 7) seems not to have any influence on the induced optical activity.

Binding constants. The calculation of the binding constants has been based on data from continuous CD titrations with the respective compounds. The technique will give precise data, which are necessary to allow a meaningful numerical analysis to be made. A prerequisite is, however, that the induced optical activity is sufficiently high in order to minimize experimental errors.

For most compounds the numerical calculation of the binding constants has given a good fit with the one-site program. When the two-site program is applied, the constant obtained for the second site is often the lowest value for K_2 in the program ($0.01 \times 10^{-4}\,M^{-1}$). A second site can not be excluded, but will under all circumstances be small. Nevertheless, this site will influence the curve-fitting procedure affecting the primary binding constants (K_1). Some ambiguities will then sometimes remain as to the exact value of K_1, but it can be definitely concluded that all the substances—with the exceptions discussed later—will bind primarily to one site on HSA at pH 7.4. The primary constants, K_1, should be close to those obtained with the one-site program.

Two groups of substances, the C_7-nitro- and the C_3-hydroxy-derivatives, deviate from the general picture described above. From the CD titration curves obtained with the nitro-derivatives it is obvious that more than one site is involved in their binding to HSA. Initially, the change of the ellipticity is comparatively small, but when more than equimolar amounts are added to the HSA solution, the ellipticity changes more rapidly. Evidently, the compounds will bind to a primary site in such a way that very small changes of the optical activity is induced, while the secondary site will induce larger changes. Unfortunately the CD titration does not given enough information for a reliable numerical analysis.

The C_3-hydroxy derivatives, Ro 5-3785 and temazepam, induced large CD changes when bound to HSA. The one-site program gave acceptable results, but the two-sites program gave inconsistent and sometimes implausible results with Ro 5-3785. With temazepam and oxazepam, the curve fitting generally was not so good as with the other compounds studied. The three-

sites program did not give further information. It is quite evident that the models applied for the numerical analysis cannot adequately describe the real situation. It is now known that oxazepam hemisuccinate is stereospecifically bound [6]. It is likely that this is true also for the other C_3-hydroxy drugs used in this study, which means e.g. that the value for the concentration of the drug in the equilibria and the programs used in the curve-fitting procedure are meaningless. This problem will be a subject for further investigations.

Acknowledgements—We thank Dr. Olle Mårtensson for valuable assistance with the calculations of the electron densities, Mrs Linnéa Wallsten for the preparation of human serum albumin and Miss A. Kober for performing the equilibrium dialysis. We also thank AB KABI, Stockholm, Roche-Produkter AB, Stockholm and The Upjohn Comp., Kalamazoo, for generous gifts of drugs. The work has been supported by the Swedish Medical Research Council (project no. 13X-3162) and by grants from Ferrosan AB, Malmö, and the I. F. Foundation for Pharmaceutical Research, Stockholm.

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