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NMR Study of the Interactions between Flurazepam and Human Serum Albumin

The Nature of the Complexation Site on the Benzodiazepin Molecule

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SUMMARY

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The nature of the interactions involved in the complexation process between flurazepam and human serum albumin has been investigated by high resolution NMR. The atoms of the flurazepam molecule which make up the site of complexation are identified by proton relaxation rate observations. Calculations show that the association constant of complexation is small (60 M^{-1}) and it appears that the flurazepam molecules are held tightly in the complex. A term which evaluates the degree of perturbation of each of the protons at the time of complexation is introduced.

INTRODUCTION

Benzodiazepins, which are widely used as anxiolytic and antiepileptic drugs, show extensive binding on HSA.[1] The influence of this reversible complexation *in vivo* is manifested in the processes of distribution of the drug in the organism, in its pharmacological effect with respect to time, in its metabolism, in its competitions with other drugs (1) and in its excretion. Our interest in such phenomena has prompted us to investigate this complexation process.

Various parameters concerning the binding in the complex have already been calculated by Muller, Wollert and Sjöholm (2-4) using results from gel permeation and circular dichroism experiments. However, the precise nature of the sites of complexation on the benzodiazepin molecules themselves has never been established.

As a means to this end, we have investigated the complexation properties of flurazepam (Dalmene) (Fig. 1), one of the most water-soluble benzodiazepinone drugs known.

The technique of high resolution N.M.R. has been employed. Jardetzky and coworkers (5, 6) have fully demonstrated the utility of such methods in the study of the interactions present in intermolecular complexes. In particular, proton relaxation rates provide information about the portion of the ligand which interacts with the protein.

MATERIALS AND METHODS

Reagents. Flurazepam was obtained from Hoffman LaRoche (Bâle). HSA of "dried purified" quality was purchased from Sochibo (Koch Light products). A sample which was extensively dialyzed against EDTA to remove all possible traces

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[1] The abbreviation used is: HSA, human serum albumin.

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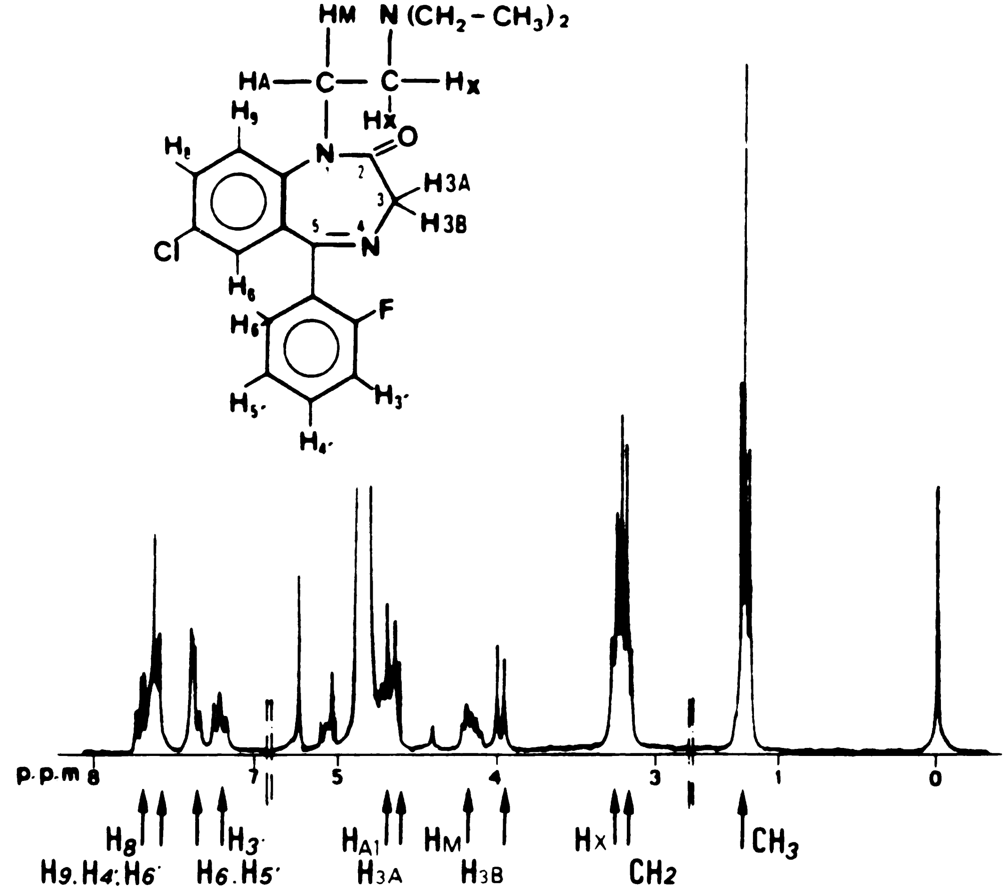

FIG. 1. *Flurazepam NMR spectra*

of metallic contamination exhibited the same proton relaxation times as the original product.

Material. Fourier Transform proton magnetic resonance spectra were recorded on a Cameca Spectrometer at 250 MHz. A 90° excitation pulse and a recovery time of 2 sec were employed.

Experimental conditions. All experiments were performed at room temperature. The ionic strength was maintained at a constant value of 0.15 by a phosphate buffer. The pH remained constant at 6.5 in order to avoid a precipitation of the drug which occurs at pH > 6.5. The flurazepam was made up into concentrations of 1.25, 2.5, 5.0 and 10.0 × 10^{-3} M while the ratios between the quantities of albumin and flurazepam were 5 × 10^{-3}, 10^{-2} and 2 × 10^{-2}. Solutions freshly prepared in water were lyophylized and then taken up again in D_2O.

The values of $1/T_2$ are calculated from the expression $(1/T_2) = \pi\Delta\nu_{1/2}$ where $\Delta\nu_{1/2}$ the full line width at half maximum height is an average taken from several spectra only when the signals are well resolved. For very broad signals an estimation of this quantity can be made by comparison with spectra of different linewidths, previously simulated by the ITERCAL[2] computer program.

Methods. Jardetzky (5) has shown that at the fast exchange limit and if there is only one complexation site on the macromolecule which is the case for flurazepam (2)

$$\frac{1}{T_{2\text{obs}}} = \frac{1}{T_{2\text{free}}} + \alpha\left(\frac{1}{T_{2\text{bound}}} - \frac{1}{T_{2\text{free}}}\right) \quad (1)$$

where $(1/T_{2\text{obs}})$, $(1/T_{2\text{free}})$ and $(1/T_{2\text{bound}})$ are, respectively, the observed relaxation

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[2] Program library supplied by CAMECA.

rate and the relaxation rates of the free and bound flurazepam, and α is the fraction bound to the HSA.

Using a simple mass action model (5) in the case where α is small,

$$\text{i.e., } [F_T] \gg [\text{HSA}]$$

$$\text{then } \alpha = \frac{Ka[\text{HSA}]}{1 + Ka[F_T]}$$

where Ka is the apparent association constant of complexation and [HSA] and $[F_T]$ are the total albumin and flurazepam concentrations, respectively. Substituting into equation (1)

$$\frac{1}{T_{2\text{obs}}} = \frac{1}{T_{2\text{free}}} + \frac{Ka[\text{HSA}]}{1 + Ka[F_T]}\left(\frac{1}{T_{2\text{bound}}} - \frac{1}{T_{2\text{free}}}\right) \tag{2}$$

or $(1/T_{2\text{obs}}) = (1/T_{2\text{free}}) + A$ [HSA] if $[F_T]$ is a constant value. A was calculated as a function of HSA concentration for each proton and at each flurazepam concentration by means of the least square method. The straight line A^{-1}, as a function of the flurazepam concentration, exhibits a slope

$$B = \left(\frac{1}{T_{2\text{bound}}} - \frac{1}{T_{2\text{free}}}\right)$$

and intercepts the Y axis at

$$C = \frac{B}{Ka}$$

Thus, the values $(1/T_{2\text{bound}})$ and Ka are easily determined. The values for the $(T_{2\text{free}}/T_{2\text{bound}})$ ratio for each proton are then studied. We wish to demonstrate that the broadening of the proton NMR signals is due to a specific interaction of the flurazepam with the protein molecules; to this end, we must show that $1/T_{2\text{obs}}$ decreases while the concentration in flurazepam increases for constant HSA concentrations.

TABLE 1

Flurazepam spectral parameters

	δ ppm	$1/T_2\ s^{-1}$		J_{Hz}
H_8	7.72	4.36	H_6–H_8	2.3
H_9	7.61		H_8–H_9	9.1
$H_{4'}$	7.60		$H_{3'}$–F	10.6
$H_{6'}$	7.60		$H_{3'}$–$H_{4'}$	8.3
H_6	7.39		$H_{4'}$–$H_{5'}$	7.3
$H_{5'}$	7.38		$H_{5'}$–$H_{6'}$	7.3
$H_{3'}$	7.21	7.10	H_{3a}–H_{3B}	11.4
H_A	4.70		H_A–H_M	14.4
H_{3A}	4.67		H_A–H_X	7.2
H_M	4.16	9.95	H_M–H_X	7.2
H_{3B}	3.99	5.65	CH_2–CH_3	7.6
H_X	3.25			
CH_2	3.20	6.16		
CH_3	1.22	4.75		

Effectively, equation (2) can be written

$$\left(\frac{1}{T_{2\text{obs}}} - \frac{1}{T_{2\text{free}}}\right)^{-1} = \left(Ka\,[\text{HSA}]\left[\frac{1}{T_{2\text{bound}}} - \frac{1}{T_{2\text{free}}}\right]\right)^{-1} + [F_T] \times \left([\text{HSA}]\left[\frac{1}{T_{2\text{bound}}} - \frac{1}{T_{2\text{free}}}\right]\right)^{-1} \tag{3}$$

Therefore, one can draw the corresponding straight line plots as a function of the flurazepam concentration for each HSA value.

RESULTS AND DISCUSSION

The NMR spectrum of flurazepam is given in Fig. 1. The chemical shifts with respect to the reference sample DSS, the coupling constants and the rates of proton relaxation of the free flurazepam at a concentration of 10^{-2} M are presented in Table 1. Resonance assignment was done by comparison with other benzodiazepin spectra (7) and by decoupling. Second order spectra δ and J values were estimated with computer simulated spectra.

The slowing down of the configurational inversion process caused by the presence of a substituent at position 1 renders the protons H_3 non-equivalent (7, 8). Effectively, these protons exhibit an AB-type spectrum. Similarly, the methylenic protons of the side-chain give rise to an AMX_2 type spectrum (8).

Unfortunately, the accidental superposition of signals at ca. δ7.6 for protons 9, 4′ and 6′ and of those at δ7.3 for protons 6 and 5′ precluded relaxation rate measurements of these nuclei. Similarly, protons 3A and A give rise to signals near to a solvent peak while the signals assigned to the methylenic protons superimpose on that of H_x.

In our measurements we have not observed any chemical shift of the drug lines upon addition of the protein to the solution.

Relaxation rate determinations. Inspection of Fig. 2 indicates that the broadening of the proton signals is produced by a specific interaction with HSA since $1/T_{2obs}$ decreases for increasing flurazepam concentrations. Elsewhere, Fig. 3 represents the variations of $1/T_{2obs}$ for several protons as a function of the temperature and appears to be in favor of rapid exchange. Apparently, an increase in the temperature leads to decreasing $1/T_{2obs}$ values.

The variation of several signal line widths with respect to HSA concentration for constant (10^{-2} M) flurazepam concentrations are plotted in Fig. 4. The straight lines corresponding to equation (2) are traced and it is observed that the one for proton 3B exhibits the greatest slope.

The resulting values A/T_{2bound}, Ka and T_{2free}/T_{2bound} of protons H_8, $H_{3'}$, H_M, H_{3B}, CH_2 and CH_3 are listed in Table 2.

The average value of the association constant of complexation is found to be 68 M^{-1}, in good agreement with results obtained by microcalorimetric methods ($Ka = 30 \pm 10\ M^{-1}$) (9). The T_{2free}/T_{2bound} values indicate that, on complexation, H_{3B} is the most perturbed proton. H_8 and H_M experience successively smaller perturbations. These findings suggest that flurazepam is bound primarily through the heterocyclic function close to N4 and then by the chlorinated ring. The high values observed for the proton relaxation rates indicate that

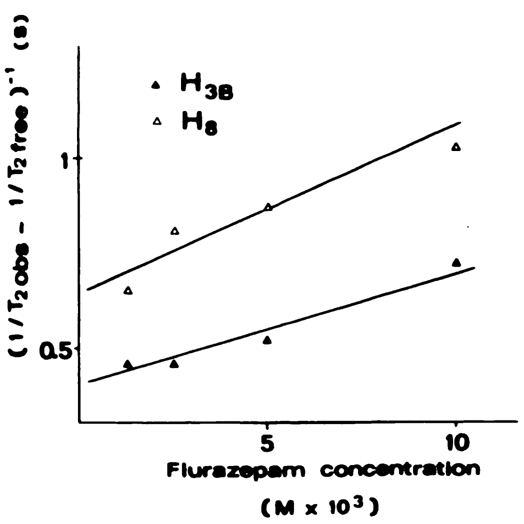

Fig. 2. *Flurazepam concentration effects on 3B and 8 proton relaxation time*
[HSA] = 2.5 10^{-5} M.

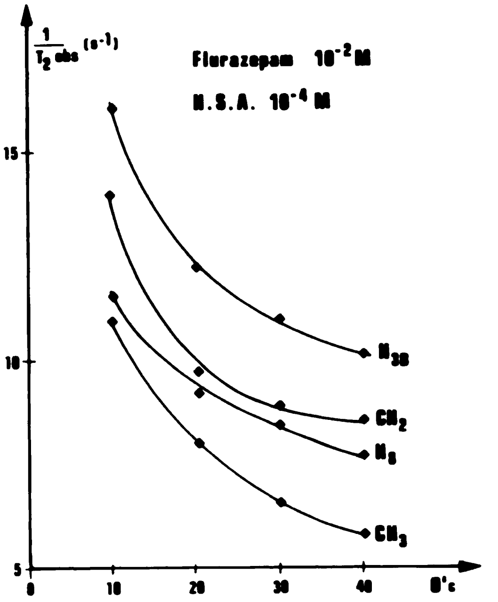

Fig. 3. *Relaxation rates of some protons in the presence of HSA as a function of temperature*

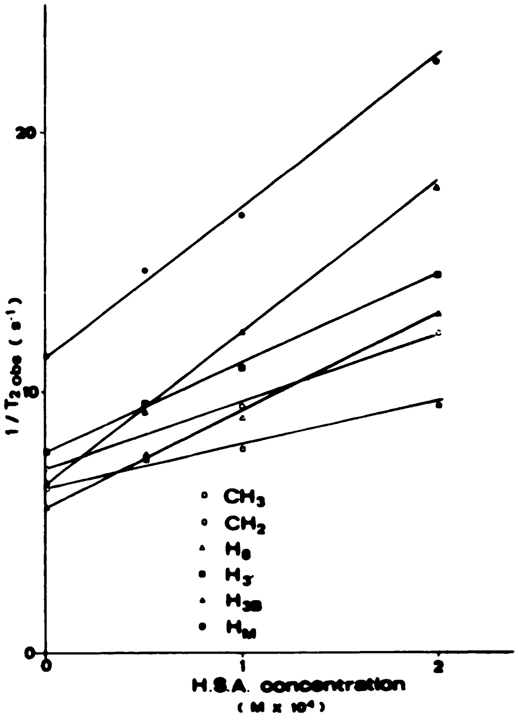

Fig. 4. *HSA concentration effects on proton relaxation time of a 10^{-2} M flurazepam solution*

TABLE 2

Binding parameters concerning each proton

	$A \times 10^{-4}$				$B \times 10^3$	$C \times 10^5$	K_A	$\frac{1}{T_{2\text{bound}}}$	$\frac{T_{2\text{ free}}}{T_{2\text{ bound}}}$
$[F_{tot}]$	10^{-2}	$5\ 10^{-3}$	$2.5\ 10^{-3}$	$1.25\ 10^{-3}$					
					s	*Mole* s^{-1}	*Mole* $^{-1}$	s^{-1}	
H_8	3.67	4.31	5.10	6.06	1.16	1.61	72	858	197
H_3'	3.44	4.24	4.83	5.24	1.13	1.77	64	883	124
H_M	5.86	7.29	8.88	9.09	0.72	0.98	73	1386	139
$H3_B$	5.88	6.83	8.68	8.53	0.65	1.06	62	1527	270
CH_2	2.57	3.36	3.73	4.11	1.62	2.22	73	614	100
CH_3	1.67	2.67	2.38	2.55	2.36	3.62	65	423	89

the flurazepam molecules are very tightly bound at the time of complexation with HSA.

However, the information gained from the T_2 measurements do not give us a full understanding of the effect of the observed $T_{2\text{bound}}$ values by the serum albumin and therefore of the interaction of the latter with each of the protons. To the first approximation, the relaxation rate of proton i can be written (10, 11):

$$\left(\frac{1}{T_2}\right)_i = M\,\tau_c \sum_j (r_{i,j})^{-6} \qquad (4)$$

where $r_{i,j}$ is the average intramolecular distance between the proton i and j, M is a constant and τ_c the correlation time. This equation implies that the principal contribution to the relaxation times is due to dipole-dipole interactions and that the value of τ_c is identical for all protons.

For a given pair of proton i and k, one can write

$$\frac{(T_2)_k}{(T_2)_i} = \frac{\sum (r_{i,j})^{-6}}{\sum (r_{k,j})^{-6}} \qquad (5)$$

where the distances $r_{i,j}$ on the benzodiazepin ring have already been estimated by Camerman (12). In the case of the side chain, $(r_{i,j})^{-6}$ values are the sum of the H-C-H interactions and of the average of all possible CH_2-CH_3 staggered interactions. These interactions are calculated with standard bound lengths and angles.

Proton H_8 is taken as the point of reference in expression (5).

Using this equation one observes, for the free flurazepam molecules, a good agreement between the T_2 expressions and the values for the internuclear distance ratios except for protons CH_2 and CH_3 which exhibit a rotation about a symmetry axis (see Table 3). One can therefore conclude that the approximations, proposed for the geometry of the system and the assumption that the correlation times τ_c are identical for all protons, are justified. Correlation time of each proton is calculated from equation (4) and the mean value is found equal to 8.2 10^{-10} S.

TABLE 3

Theoretical and experimental relaxation rate ratios

	$\Sigma(r_{i,j})^{-6} \times 10^3$	$\Sigma(r_{i,j})^{-6}$ ratio	$T_{2\text{ free}}$ ratio	$T_{2\text{ bound}}$ ratio	$\tau_{c\text{ free}} \times 10^{10}$
	Å^{-6}				(*S*)
H_8	5.73	1.0	1.0	1.0	8.6
H_3'	10.25	1.79	1.63	1.03	8.1
H_M	12.66	2.21	2.28	1.59	9.2
H_{3B}	7.13	1.25	1.30	1.77	9.3
CH_2	40.51	7.07	1.41	0.72	1.8 7.1[a]
CH_3	31.83	5.55	1.09	0.50	1.7 6.9[a]

[a] Corrected for internal rotation (13).

Nitrogen 14 quadripolar effects were not taken into account. Also, uncertainty as to the position of the side chain protons with respect to aromatic protons prevents the calculation of these interactions. These two effects may significantly decrease the correlation time.

However, the value for the $T_{2\text{bound}}$ and the internuclear distance ratios exhibit no agreement. Moreover equation (4) gives a mean bound correlation time equal to 1.6 10^{-7} S, a value which is too high. It appears that the hydrogens are broadened mainly by adjacent protein hydrogens (14). Therefore it is necessary to add a new term, I_i, to the $(r_{i,j})^{-6}$ intramolecular expression which acknowledges these interactions. As a result, equation (4) becomes

$$\left(\frac{1}{T_{2\mathrm{bound}}}\right)_i = M\tau_{c_{\mathrm{bound}}}\left(I_i + \sum(r_{i,j})^{-6}\right)$$

where $\tau_{c_{\mathrm{bound}}}$ and I_i are unknown. Moreover, one can suppose that, to the first approximation, the correlation time of the flurazepam bound to the protein is only slightly different from that of the latter molecule. That is,

$$\tau_{c_{\mathrm{bound\ flurazepam}}} = \tau_{c_{\mathrm{HSA}}}$$

Now, it is possible to calculate these values from the relationship

$$\tau = \frac{4\pi\eta r^3}{3\mathrm{KT}}$$

where η is the viscosity of the solution and r is the radius of the spherical molecule (10). If one assumes that the HSA approximates a spherical molecule, then the radius of such a species would be 31 Å (15). The viscosity of the solutions used in the experiments was found to be of the order of 1.02 × 10^{-2} poise. On substituting these values into the equations above, one calculates a value of 3.1 × 10^{-8} S for τ_c, in good agreement with the figure calculated by Wallach (16) for BSA.

Substituting the value for τ_c into the expression derived from equation (4), it is possible to calculate the values of I_i for each proton (Table 4).

For CH_2 and CH_3 protons, $T_{2\mathrm{bound}}$ is dependent on the rotation about the symmetry axis and on the random tumbling of the axis. The relative contribution of the two interactions cannot be accurately calculated. However, if we suppose a very fast rotation of these groups we may find a limit value for I_i. So the true value will be lower.

On the other hand it is found that protons H_3, H_M and H_8 exhibit the largest values for this parameter. These protons are, therefore, the most tightly held in the complex. This result is in good agreement with the $\frac{T_{2\mathrm{free}}}{T_{2\mathrm{bound}}}$ ratios although there is a reversal in the order of the values for H_M and H_8. It seems, therefore, that this expression is not directly proportional to the parameter I_i, but a function of the ratio $I_i/(r_{i,j})^{-6}$. Therefore one can write

TABLE 4

Estimated values of HSA interaction with each proton

	H_8	H_3'	H_M	H_{3B}	CH_2	CH_3
$I_i \times 10^2$	2.67	2.31	3.97	5.06	5.22	3.21

$$\frac{T_{2\mathrm{free}}}{T_{2\mathrm{bound}}} = K\left(1 + \frac{I_i}{r_{(i,j)}^{-6}}\right)$$

CONCLUSION

We have demonstrated that the flurazepam binds itself to HSA by its heterocyclic component close to the nitrogen atom N_4. It is reasonable to state that this is also the case for other benzodiazepins which contain the same heterocyclic structure. This conclusion appears justified in the light of work carried out elsewhere (17), which suggests the presence of cationic binding sites on the HSA. The heterocyclic moiety of benzodiazepin is negatively charged as has been demonstrated from electron densities computed by the CNDO/2 method (18). Such features present in flurazepam and HSA lead us to propose the existence of electrostatic-type bonding between the two systems. Whereas the benzene ring seems to play the role of a secondary hydrophobic site of complexation, the fluorinated ring does not appear to enter into such processes. The sole effect of this group is to influence the charge present on the nitrogen atom N_4 (18).

It seems likely that the positive side chain hinders the bonding between the flurazepam and HSA resulting in a low value for the association constant of complexation and an exalted value of the interaction term, I_i, for proton H_M.

This work has demonstrated the marked utility of such NMR methods with respect to other techniques in the field of weak drug-protein interactions.

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