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SHORT COMMUNICATIONS

A study on the binding of diazepam to serum albumins by T_1 NMR measurements

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It is well known that diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-3H-1,4-benzodiazepin-2-one) binds to serum albumin: various authors [1–8] have proved the presence of one specific binding site. The binding constants, the thermodynamic parameters and their variation with pH have also been determined [9, 10]. Correlations between binding strength and electron distribution [11], N–B transition of albumin and diazepam binding [12], and also between biological activity and benzodiazepine stereochemistry [13–15] have been proposed. Numerous binding sites have been hypothesized on the protein molecule [16, 17]. A study by Sarrazin *et al.* [5] hypothesized N(4) as a binding site of benzodiazepines, on the basis of changes in the width of protonic signals, which can be approximately correlated with the T_2 relaxation times. The aim of our work was to provide evidence of the binding site on benzodiazepines by analysing, according to Jardetsky [18], how HSA (human serum albumin) and BSA (bovine serum albumin) affect both the ^{13}C and the ^{1}H T_1 relaxation times of the heterocyclic moiety of benzodiazepines; the analysis of the ^{13}C in addition to the ^{1}H relaxation times should give a deeper insight into the binding mechanism.

The comparative analysis of the behaviour of diazepam with BSA and HSA supplies an explanation of the binding differences remarked by Müller and Wollert [7].

Materials and methods

Reagents. Diazepam was supplied by Roche (Milan, Italy) and was used without further purification. BSA and HSA were obtained from Sigma Chemical Co. and purified according to McMenamy and Oncley [19] and Chen [20].

Instruments. A Cary 219 UV–visible spectrophotometer was used in the spectrophotometric determination of protein concentration. The determination of relaxation times (T_1) was performed on a Varian CFT-20 spectrometer.

Methods. The relaxation times of solutions of diazepam in $CDCl_3$ (3.5×10^{-2} M for ^{1}H and 3.5×10^{-1} M for ^{13}C) were determined either in the absence or presence of added BSA (or HSA) (1.4×10^{-6} M). The determination of T_1 was performed by using the (180–τ–90–T) pulse sequence. The values of T_1 for C(2) and C(5) were determined by the relation $T_1 = \tau/\ln 2$, where τ is the interval between the perturbing and observing pulses, because of the very long relaxation times; the T_1 values for the other carbon atoms and protons were determined by least-squares regression of the relation $\ln |M_0 - M(\tau)|/M_0 = -\tau/T_1$. The anomalous high value of T_1 for the C(1) carbon atom can be explained by taking into account the dipole–dipole relaxation time. This can be calculated from the nuclear Overhauser effect, whose value η was estimated from $\eta = M(\text{decoupled})/M(\text{suppressed}) - 1 = \eta_0 \times T_1/T_{DD}$ (where $\eta_0 = 1.988$). Our $T_{DD} = 6.9$ value is indicative of a great contribution of the dipole–dipole interaction [21].

Before calculating the relaxation times, we analysed the protonic and ^{13}C spectra of diazepam in $CDCl_3$. The spectra are shown in Fig. 1 and the spectral parameters are reported in Table 1. The relaxation times for the overlapping aromatic resonances of (6), (8), (9) and (13), (14), (15), (16) and (17) protons were roughly estimated from band-widths by using a non-linear least-squares programme in Basic for the Hewlett–Packard HP-85 computer.

The serum albumins were checked before and after NMR experiments by polyacrylamide gel electrophoresis in Tris–glycine at pH 8.3, and they always showed the same pattern.

Table 1

Carbon number, chemical shift and multiplicity*					
1′	34.7 *q*	6	129.8	11	129.1
2	169.8 *s*	7	130.0	12	138.1 *s*
3	56.9 *t*	8	131.4	13,17	129.4
5	168.8 *s*	9	122.5	14, 16	128.3
		10	142.5 *s*	15	130.6
Proton number, chemical shift and coupling constants†					
1′	269.2	6	578.1	13, 17	600.5
3a	383.2	8	592.0	14, 16	582.3
3b	300.0	9	592.0	15	584.5
$J_{3a,3b}$	10.8	$J_{6,8}$	2.6	$J_{13,14}$	8.2
		$J_{6,9}$	0.3	$J_{13,15}$	0.6
		$J_{8,9}$	8.5	$J_{13,17}$	2.5
				$J_{14,15}$	7.5
				$J_{14,16}$	2.0

* Chemical shift in ppm from TMS; multiplicity from off-resonance.

† Chemical shift (in hertz from TMS) and coupling constants were optimized by the programme LAOCOON III [22].

Results and discussion

The choice of an organic solvent was necessary due to the very low solubility (for these kind of measurements) of diazepam in water. On the other hand, supported by the interesting hypothesis that the degree of binding can be correlated with conformational states of serum albumin [12], we supposed that the binding sites (try, his, arg, tyr) could be more accessible to diazepam in $CDCl_3$ than in water; if this was the case, the binding groups of diazepam should be much clearer.

In Table 2 we report the calculated values of relaxation times for pure diazepam, in the presence of BSA and in the presence of HSA. An inspection of the reported values clearly shows that the binding to both albumins, in the heterocyclic moiety of diazepam, influenced primarily C(5) and in a decreasing way C(3), H(3) and C(2), while C(1) and H(1) were completely unaffected.

On the other hand, with respect to BSA, HSA had a very different effect on the protons of the B-aromatic ring: in fact their relaxation times were altered by BSA but not by HSA.

These results suggest two different mechanisms of binding for HSA and BSA, in agreement with the findings of other authors [7]. In particular, they allow us to hypothesize: (i) a strong specific electrostatic binding between N(4) on diazepam and different groups on HSA; and (ii) an interaction between diazepam and a non-specific binding

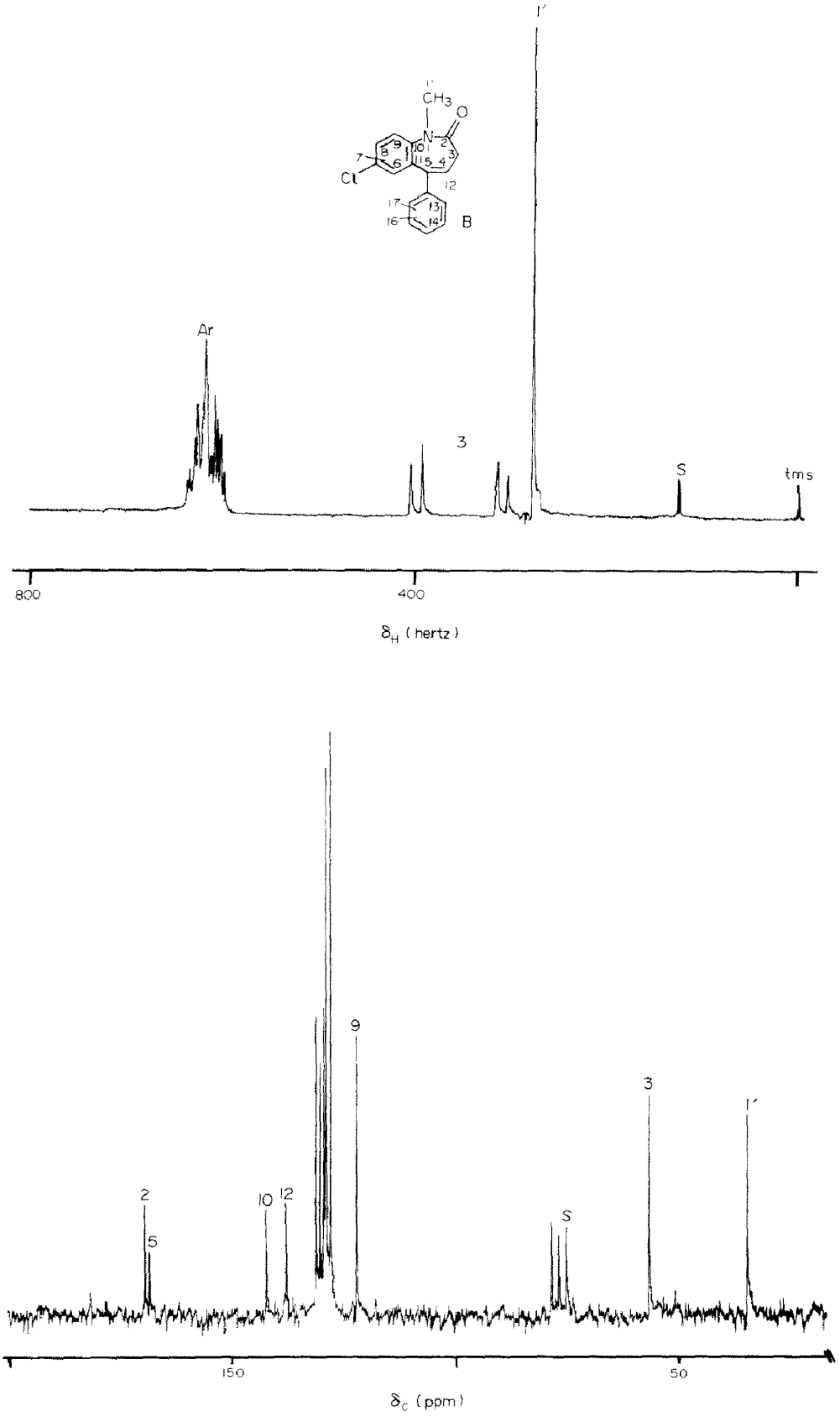

Fig. 1. ^{1}H NMR (upper trace) and ^{13}C NMR of diazepam in $CDCl_3$ (lower trace).

Table 2. Carbon and proton number and T_1 relaxation times (sec)

	Diazepam	Diazepam + BSA	Diazepam + HSA
C(1′)	3.7	5.5	5.8
C(2)	16.0	14.0	13.0
C(3)	0.76	0.65	0.58
C(5)	17.0	10.0	8.0
H(1′)	1.52	1.55	1.59
H(3)	1.08	0.9	0.8
H(13–17)*	1.2	0.4	1.1

* Relaxation times estimated from half band-width.

site on BSA through the B-aromatic ring; this last interaction likely weakens the electrostatic binding to N(4) for steric hindrance.

These results on a single molecule obviously cannot be generalized, but nevertheless they point out the utility of such an approach in obtaining an insight into the binding mechanism of benzodiazepines to serum albumins.

In summary, the binding of diazepam to bovine and human serum albumin has been studied by measurements of ^{13}C and ^{1}H NMR spin lattice relaxation times in $CDCl_3$ solutions. The comparison of the T_1 values of carbons and protons of the heterocyclic moiety of the diazepam molecule in the presence of albumins, with those obtained for pure diazepam allows us to point out that both proteins affect the C(3) and C(5) carbons and the H(3) proton. These results clearly support the presence of an electrostatic interaction between N(4) and one cationic site on the serum albumins. Furthermore, a very different behaviour for BSA amd HSA is evident for the aromatic protons of the B-ring, suggesting that this group determines the difference in binding found by other authors.

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