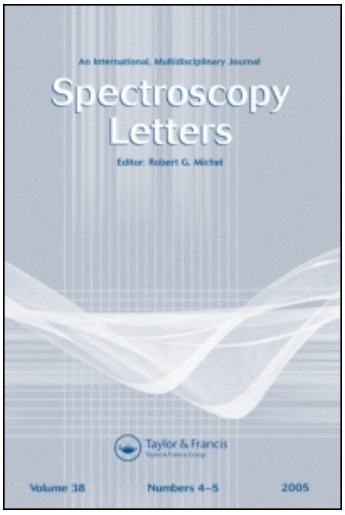

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STUDY OF THE INTERACTION BETWEEN TRYPANOCIDE 3-NITROBENZALDEHYDE GUANYL HYDRAZONE AND BOVINE SERUM ALBUMIN BY EQUILIBRIUM DIALYSIS AND NMR

Key words: 3-nitrobenzaldehyde guanyl hydrazone, Equilibrium dialysis, NMR spin-lattice relaxation time.

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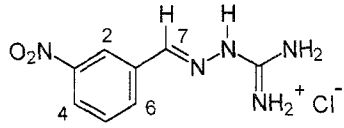
ABSTRACT: Equilibrium dialysis and NMR spin-lattice relaxation time (T_1) studies of the interaction of the trypanocidal drug 3-nitrobenzaldehyde guanyl hydrazone (3NBGH) with bovine serum albumin (BSA) were used to show that this interaction is weak and nonspecific, making 3NBGH a potential agent for the prophylaxis of *Trypanosoma cruzi* infected blood.

INTRODUCTION

Trypanosoma cruzi, the causative agent of Chagas Disease, is responsible for about 45,000 deaths per year in South and Central

America.[1] The transfusion of blood contaminated with *T. cruzi* is the second most important way of infection of this disease in endemic areas, and the only way of contagion in countries where Chagas Disease is unknown.[2,3] Although the detection of *T. cruzi* is cheap and very simple, the probability of receiving a blood transfusion with blood tainted with this protozoan can be as high as 2.19% in certain regions of South America.[4] Even in countries outside the endemic zone, the combination of immigration from endemic areas, blood transfusion and the absence of blood testing for *T. cruzi* could lead to spreading of this disease.[2] For example, it is believed that, of the several million United States citizens of Latin American origin, about 100,000 are infected with this parasite,[5] a situation that may be responsible for a few cases of the disease in that country.[6,7] The drug actually used in blood prophylaxy regarding *T. cruzi* is gentian violet, which however efficient may cause some adverse effects to the erythrocytes.[8] Therefore, the development of new drugs for the disinfection of collected blood in blood banks may be an important step towards the elimination of this form of contagion and the control of this terrible disease.

Our previous studies with guanyl hydrazones showed that these compounds are active against the trypomastigote forms of *Trypanosoma cruzi* in infected blood, and that this activity is not associated with greater toxicity for the red cells.[9] The mechanism of action of these compounds is still unknown, but it has been suggested that it may be related to the interaction of the drugs with the DNA or the cell membrane of the parasite.[10] In order to determine the possibilities of the guanyl hydrazones as drugs for blood prophylaxy, it became necessary, among other studies, to determine their effect on blood proteins. In this work, we study the interaction of 3-nitrobenzaldeyde guanyl hydrazone (3NBGH, Fig. 1) with bovine serum albumin (BSA) using equilibrium dialysis and



3NBGH

FIG. 1: 3-Nitrobenzadeyde Guanyl Hydrazone.

NMR. This compound, which is more active *in vitro* (ID_{50} 182.6 μM) than gentian violet (ID_{50} 536 μM), is a potential agent for the prophylaxy of *T. cruzi* infected blood.

RESULTS AND DISCUSSION

The dialysis experiments were carried out according to standard procedures[11] using 1 mL Scienceware dialysis cells at a temperature of 37.0 ± 0.1 °C, and the data were treated as proposed by Klotz[12] and Scatchard.[13] Five 3NBGH concentrations (100, 80, 65, 50 and 35 μM) were used, with each experiment being repeated three times. A buffer solution with pH close to 7.0 would be the ideal solvent for these experiments, however, the use of buffers of phosphate or bisulfate led to precipitation of the drug. Alternatively, a 0.1 M acetate buffer (pH 5.0), which had been previously utilized for studies of this kind,[14] was used. The equilibration time was determined from a blank run with the 100 μM solution of 3NBGH for 24 hours, and the concentration of BSA used was either 7.25×10^{-4} M or 7.25×10^{-5} M. The free drug concentration after equilibration (**Df**) was determined from the residual drug concentration in the chamber without protein, using the absorbance of the solution at 273 nm. The number of mols of the drug bound to 1 mol of the BSA (**r**) was calculated from the decrease in drug concentration.

The experiments with the lower BSA concentrations were difficult to reproduce and less precise. Fortunately, the plot of **1/Df** *vs.* **1/r**, for a concentration of BSA 7.25 x 10^{-4} M, produced the straight line shown in Figure 2.

According to these results, the binding constant for this interaction (**K**) is 2.03 x 10^{4} M^{-1}, indicating that 3NBGH would have a relatively strong affinity for BSA. On the other hand, the same results yield a number of 3NBGH binding sites per molecule of BSA (**n**) of only 0.02, accounting for the very small changes in drug concentration observed in all the dialysis experiments. If **n** were 1 or 2 we would have observed greater changes in drug concentration upon equilibration. With these results it is difficult to reach a clear conclusion regarding the BSA-3NBGH interaction, but considering the calculated values of **K** and **n** together, it seems more reasonable to consider this interaction as weak.

A very sensitive way to study drug-biomolecule interactions is the measurement of the relaxation times of the drug hydrogens in solutions of the pure drug and in the presence of the biomolecule. Both, spin-spin (T_2) and spin-lattice (T_1) relaxation times are dependent on the molecular correlation time and sensitive to changes in the solvating environment.[15] In this work we used T_1 monitoring to study the degree and topology of association for the system 3NBGH-BSA. All the experiments were carried out in a Varian Unity-300 NMR spectrometer with careful temperature regulation at 37 ± 0.1 °C and using TMS in a capillary as external reference. The spin-lattice relaxation times (T_1) were measured using the inversion recovery (IR) pulse sequence,[16] using relaxation delays varying between 12 and 32 s, depending on the values of T_1 and on the sample concentration. The values of τ (the second delay on the IR sequence) varied from 12.5 ms to 32 s. The reference samples were prepared by dissolving 3NBGH in 0.1 M acetate buffer in D_2O (pD 4.75).

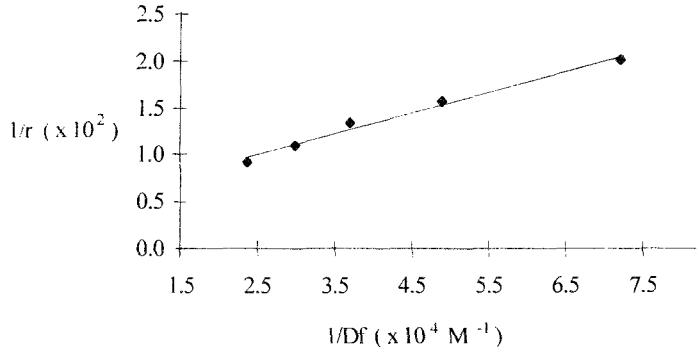


FIG. 2: Scatchard plot for the binding of 3NBGH to BSA (7.25×10^{-4} M) in 0.1 M acetate buffer at 37 °C. In the figure: $1/r = 1/n + 1/nK$ $(1/Df) \rightarrow 1/r = 44.673 + 0.00221(1/Df)$ and $R^2 = 0.991$.

For the interaction studies, the desired amount of 3NBGH was dissolved in a solution of BSA in 0.1M acetate buffer in D_2O. The BSA concentration used in the assays was chosen as 7.25×10^{-5} M from Figure 3, as this value leads to greater variation of T_1 while maintaining different T_1 values for the different hydrogens. The results are shown in Tables 1 and 2.

The increase in the concentration of 3NBGH in the samples without BSA produces a gradual decrease on the T_1 values of all the hydrogens. This effect, which is seen more clearly in Figure 4a, is due to the association of the 3NBGH molecules at higher concentrations. The confirmation of this associative behavior of 3NBGH was obtained by the observed NMR line width narrowing and the T_1 increase produced by the elevation of the sample temperature.

The presence of BSA attenuates the variation of T_1 with drug concentration (Figure 4b). This result indicates the existence of an interaction between 3NBGH and BSA, and that the interaction is unspecific. The hydrogen atoms that are affected in higher degree by the

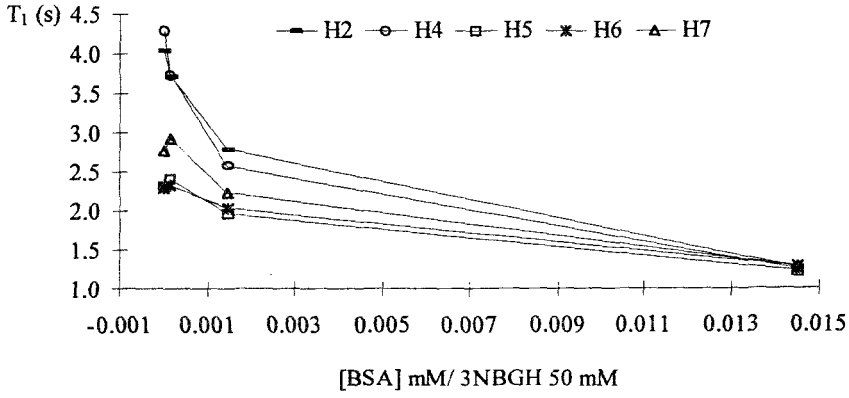


FIG. 3: Variation of T_1 in a 50 mM solution of 3NBGH with the concentration of BSA.

presence of BSA are the ones in *ortho* position to the nitro group, followed by the side chain hydrogen (H7) (Figure 5), suggesting that the interaction of 3NBGH with BSA occurs first, through the nitro group and secondly, in a weaker manner, through the cationic side chain.

To eliminate the possible competitive effect of the buffer with 3NBGH for the interaction sites of BSA, the experiments were also run in pure D_2O. The results showed that the 3NBGH-BSA interaction is not significantly affected by the presence of the buffer in the solutions used, as the curves in D_2O are very similar to the ones in acetate buffer. Our results indicate that the decrease of T_1 with the increase in drug concentration is caused by the intermolecular drug-drug interaction. This interaction interferes with the binding of the 3NBGH on BSA because the reduction in T_1 with the increase in drug concentration at constant protein concentration is more pronounced at low drug concentrations. The large T_1 diminution with constant 3NBGH concentration (50 mM) and increase in BSA concentration is not due to the influence of viscosity

TABLE 1

T_1 (in seconds) for the Non-Exchangeable Hydrogens of 3NBGH at Different Concentrations in 0.1 M Acetate Buffer.

	Concentration of 3NBGH (mM)			
Atom	13	25	50	75
H2	5.4±0.4	4.4±0.2	4.0±0.2	4.0±0.3
H4	5.3±0.5	4.6±0.2	4.3±0.3	3.8±0.4
H5	2.9±0.4	2.7±0.3	2.3±0.1	2.3±0.3
H6	2.9±0.2	2.7±0.2	2.3±0.1	2.3±0.1
H7	3.7±0.4	3.3±0.4	2.8±0.2	3.1±0.5

TABLE 2

T_1 (in seconds) for the Non-Exchangeable Hydrogens of 3NBGH at Different Concentrations in 0.1 M Acetate Buffer with BSA 7.25×10^{-5} M.

	Concentration of 3NBGH (mM)			
Atom	13	25	50	75
H2	3.0±0.2	2.8±0.1	2.8±0.1	2.6±0.1
H4	2.8±0.2	2.7±0.2	2.6±0.2	2.5±0.1
H5	2.6±0.3	2.4±0.3	2.0±0.1	1.8±0.1
H6	2.3±0.1	2.2±0.1	2.0±0.1	1.8±0.1
H7	2.8±0.2	2.6±0.3	2.2±0.1	2.0±0.1

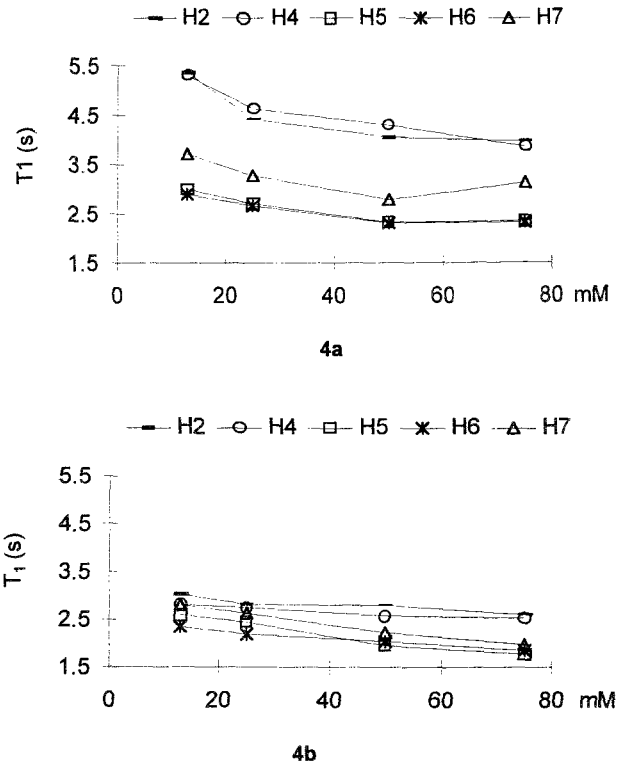


FIG. 4: Variation of T_1 with the concentration of 3NBGH in 0.1 M acetate buffer alone (Fig.4a) and in the presence of 7.25×10^{-5} M BSA (Fig. 4b).

effects on the T_1 relaxation times because all the solutions are diluted enough to ensure that the viscosity of the solution is the same as the pure solvent. As mentioned before, the greater changes of T_1 values in the presence of BSA occur with H2, H4 and H7, indicating that the 3NBGH-BSA interaction is of electrostatic nature, and that it involves principally the nitro group, with the guanyl hydrazone side chain playing a secondary role. This is represented graphically in Figure 6.

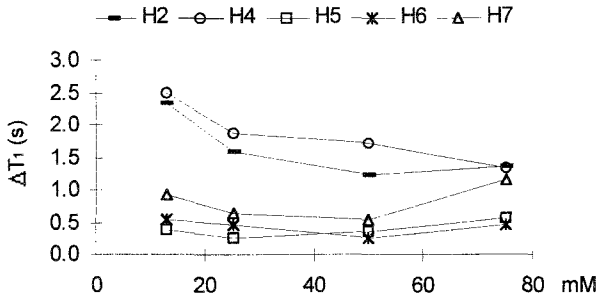
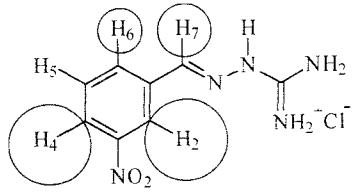


FIG. 5: Variation of ΔT_1 (pure-BSA) for the different hydrogens of 3NBGH with the concentration of 3NBGH.



3NBGH

FIG. 6: Graphic representation of the binding points of 3NBGH on BSA.

The T_1 interaction results and the value of **K** obtained by equilibrium dialysis indicate that BSA may serve to transport 3NBGH without detectable deleterious effects on the protein. Being about 2.9 times more active *in vitro* than gentian violet and non-toxic to the red cells nor deleterious to BSA in the concentrations used in this work, 3GHNB is a candidate for use in the preventive treatment of blood collected in blood banks, principally in Chagas Disease endemic zones. Other drugs of this family, some of which present ID_{50} values up to 10 times lower than 3NBGH, are being considered for use in blood prophylaxis and for the intravenous chemotherapy of Chagas Disease.

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