BINDING FEATURES OF DIACETYLMORPHINE (HEROIN) IN WHOLE BLOOD AND IN BLOOD FRACTIONS

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ABSTRACT Binding features of heroin in whole blood and in blood fractions were delineated by measuring the selective spin-lattice relaxation rates of heroin protons in physiologic conditions. Interaction with some receptor located in the whole human blood or in the human plasma was detected and the apparent binding constant calculated (K = 39 mol^{-1} dm^3). Inferences about molecular dynamics of the bound heroin could be also gained.

INTRODUCTION

One of the most intriguing problems for investigators is how biologically active effectors bind to their macromolecular receptors.

NMR techniques have been widely used to study this problem since the NMR time scale is effective in transmitting information from the bound to the bulk environment, where it can be more easily detected. This situation is usually referred to as fast exchange limit which, for the spin-lattice relaxation rates of protons within the effector molecule, yields

$$R_{1obs} = p_f R_{1f} + p_b R_{1b}, \quad (1)$$

where f and b refer to the free and bound environments, respectively and the p's are the fractions of effector molecules in the two environments.

It is easily recognized that the approach based on longitudinal proton relaxation (Eq. 1) is feasible only where $p_b R_{1b} \gg 0$ so that a R_{1obs} quite different from R_{1f} can be measured.

Since p_b must be kept very small ($p_b \ll 1$, $p_f \simeq 1$) Eq. 1 can be suitably applied only in situations where R_{1b} is very large ($R_{1b} > R_{1f}$). This has limited NMR investigations of the commonly encountered diamagnetic effector-receptor pairs; in fact, when the molecular motions slow down, well outside the extreme narrowing region ($\omega_o \tau_c \gg 1$), the values of the proton R_1 become very small in most cases (1).

Extensive applications of the NMR method were, however, shown to be possible (1–3) even in diamagnetic macromolecular systems by measuring the proton spin-lattice relaxation rates following selective excitation of properly chosen resonances within the ^{1}H NMR spectrum of the effector. In this case the relaxation rate is an almost linear function of τ_c and it becomes indefinitely large as the molecular motions slow down.

The theory underlying the occurrence of these phenomena has been thoroughly reported elsewhere (1). The measurements of selective proton spin-lattice relaxation rates (R_1^s) provided a strong investigative tool for the understanding of the binding interactions between small substrates and enzymes (1), as well as between local anesthetics and model and biological membranes, and even whole cells (2, 3).

Here, as part of our project on the physicochemical properties of morphine opiates (4, 5), we investigated binding interactions between diacetylmorphine (heroin) and blood components. It is known that, while the potential for heroin hydrolysis exists in practically every tissue, the reaction in the blood is very rapid, the half-life being a few minutes (6–9) in both dog and human blood. In spite of this, the specific binding of heroin and morphine to human serum proteins and other constituents of the blood has not been completely clarified. We have measured the selective relaxation rates of heroin aromatic protons in whole blood and various blood fractions to better understand the binding features, including the apparent association constant and the motional dynamics of heroin at the bound site.

MATERIALS AND METHODS

Diacetylmorphine hydrochloride (Supelco, Inc., Bellefonte, PA) was dissolved in a deuterated phosphate buffer. Blood samples from volunteers were withdrawn by venipuncture into heparinized syringes, and the plasma was separated from the red blood cells (RBCs) by centrifugation at 4,000 g for 15 min at 4°C. The plasma was removed by aspiration. The RBCs were washed twice by resuspending in 0.15 M NaCl 0.05 M phosphate pH 7.5 buffer, and were recovered by centrifugation. Fractionation into human serum and RBCs was carried out in a similar way.

The NMR measurements were performed with an XL-200 NMR spectrometer (Varian Associates, Inc., Palo Alto, CA) at the constant temperature of 37 ± 1°C. The nonselective proton spin-lattice relaxation rates, R^{ns}, were measured by using the inversion recovery $(\pi - \tau - \pi/2 - t)_n$ pulse sequence.

The selective proton spin-lattice relaxation rates, R^S, were measured in the initial rate approximation (10) by giving a selective π pulse with the proton decoupler at the selected frequency for a relatively long time (19–21 ms). After the time τ a nonselective $\pi/2$ pulse was given to detect

the longitudinal magnetization. The R^{nS} or R^{S} values were obtained from a three-parameter exponential regression analysis of the recovery curve for longitudinal magnetization.

RESULTS AND DISCUSSION

Typical nonselective and selective proton relaxation rate measurements are reported in Figs. 1 and 2, respectively, whereas all the data on relaxation are summarized in Table I. For a pure dipole-dipole ^1H-^1H relaxation pathway, the R^{nS} and R^{S} of any proton i are given by (11)

$$R_i^{nS} = \sum_{i \neq j} \rho_{ij} + \sum_{i \neq j} \sigma_{ij} \tag{2}$$

$$R_i^{S} = \sum_{i \neq j} \rho_{ij}, \tag{3}$$

where ρ_{ij} and σ_{ij} are the direct relaxation and the cross relaxation terms for a proton pair.

$$\rho_{ij} = \frac{1}{10} \frac{\hbar^2 \gamma_H^4}{r_{ij}^6} \left\{ \frac{3\tau_c}{1 + (\omega_0 \tau_c)^2} + \frac{6\tau_c}{1 + (2\omega_0 \tau_c)^2} + \tau_c \right\} \tag{4}$$

$$\sigma_{ij} = \frac{1}{10} \frac{\hbar^2 \gamma_H^4}{r_{ij}^6} \left\{ \frac{6\tau_c}{1 + (2\omega_0 \tau_c)^2} - \tau_c \right\}, \tag{5}$$

where r_{ij} is the proton-proton distance. It has been shown (10) that a pure dipolar mechanism yields, for a proton pair, $R^{nS}/R^{S} = 1.5 \cdot R^{nS}/R^{S}$ values lower than 1.5 are expected either outside the extreme narrowing region ($\omega_0 \tau_c \gg 1$) or whenever mechanisms other than the dipolar are effective. Since this last reason does not seem to be suitable in the case of the heroin molecule, it can be stated that the $\omega_0 \tau_c > 1$ condition applies and, therefore, the motional correlation time can be evaluated (1) ($\tau_c = 8.1 \times 10^{-10}$ s at 310°K). Such a value of the rotational correlation time agrees with the one calculated for the morphine molecule in aqueous solution (5). Moreover, the use of this value of τ_c allows the calculation of the selective relaxation rate of the H-1 proton, which is 2.45 Å apart from H_2 (12). The difference between the calculated and the experimental value was of the same order of magnitude as the experimental error.

The effects of adding blood or various blood fractions to the heroin solution are summarized in Table II. It is apparent that only upon addition of human blood or human plasma a significant enhancement of the selective proton spin-lattice relaxation rate ($\Delta R^{S} = R^{S}_{obs} - R^{S}_{blank}$) of all the low-field protons of the heroin molecule could be detected.

The observed R^{S} enhancement is a direct consequence of the slowing down of molecular motions of all or part of the heroin molecules. In other words, the binding of a fraction of heroin or viscosity effects could be, in principle, alternatively claimed to underlie the observed relaxation behavior. However, as it will be reported later, different ΔR^{S} values were measured in samples differing only in the concentration of heroin, so that viscosity changes could not be responsible for the relatively large changes in R^{S}. In fact, the largest ΔR^{S}'s were found in correspondence with the lowest heroin concentrations and vice versa (see Fig. 3). It could be therefore concluded that the R^{S} enhancement results from binding of a fraction of heroin molecules

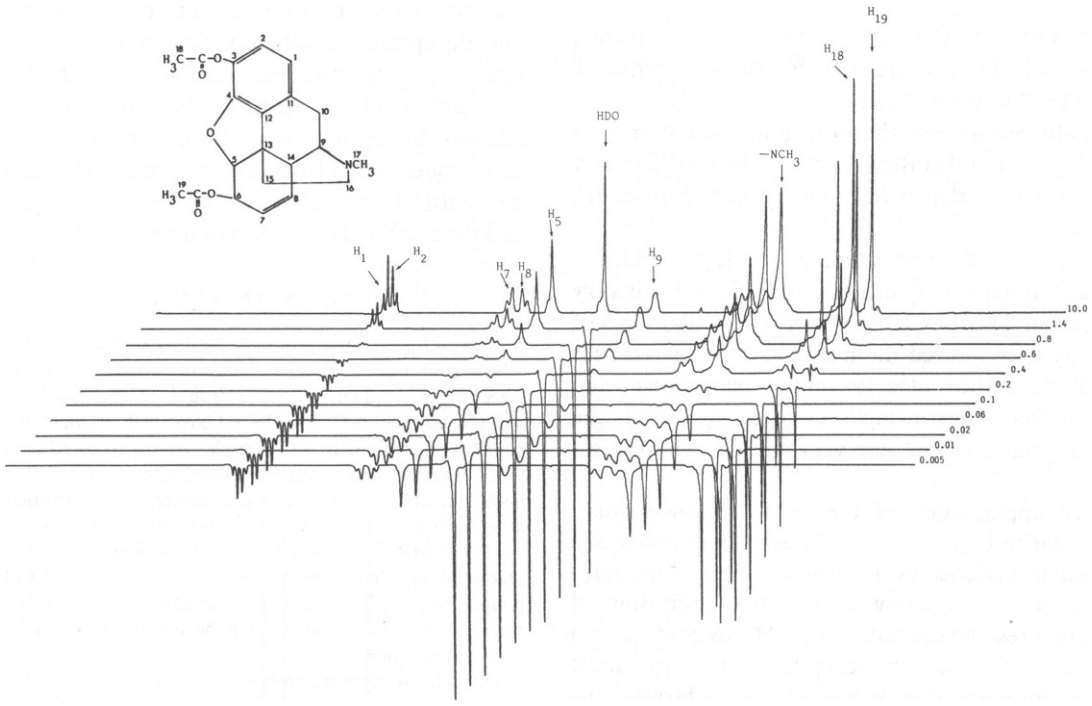

FIGURE 1 Nonselective partially relaxed proton spectra for heroin 0.1 mol dm^{-3} in physiologic deuterated buffer at pH = 6.8 and T = 310°K.

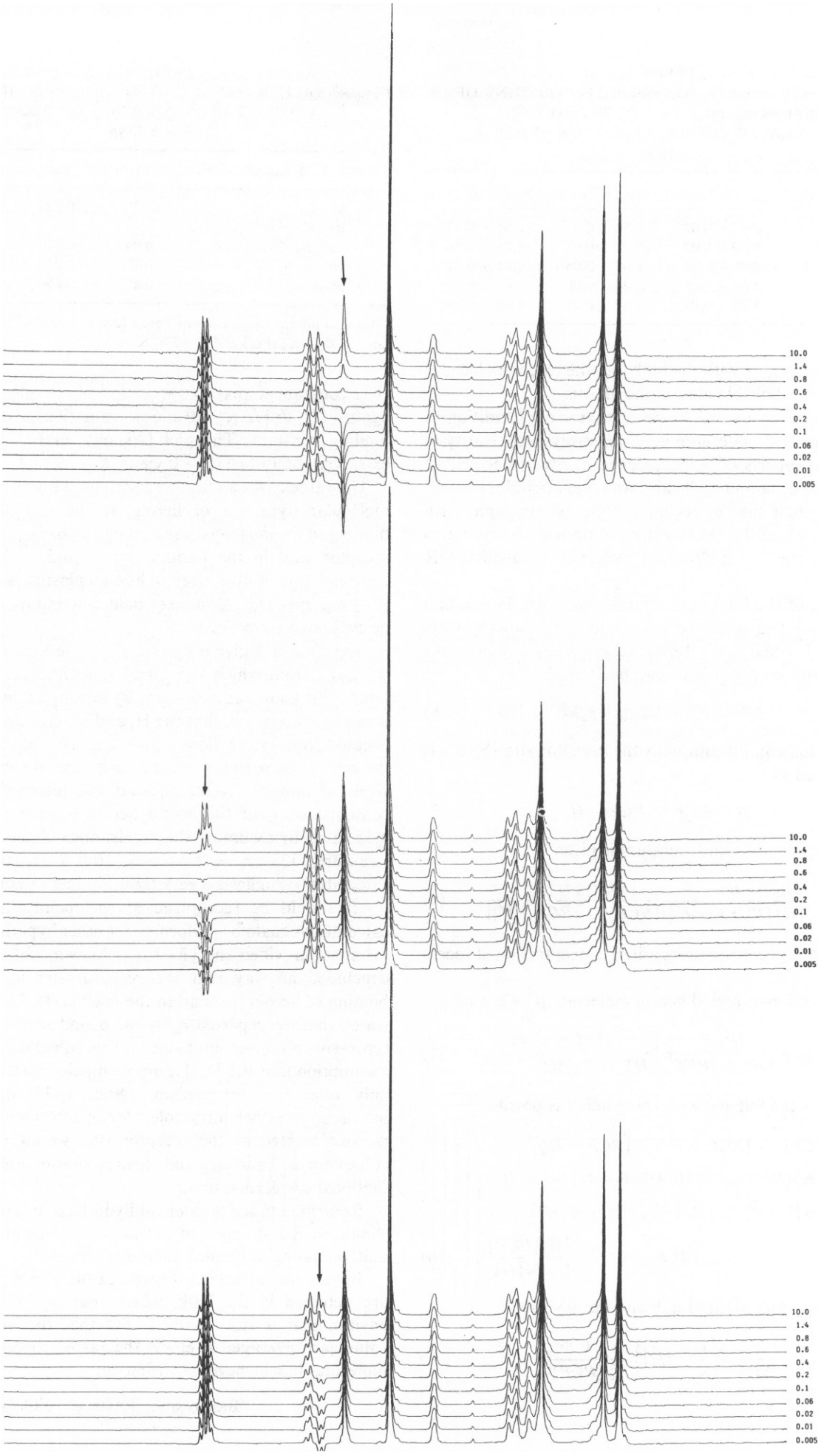

FIGURE 2 Selective partially relaxed proton spectra of heroin 0.1 mol dm^{-3} in physiologic deuterated buffer at pH = 6.8 and T = 310°K. The arrow indicated the frequency for the relative inversion.

TABLE I
RELAXATION DATA FOR SELECTED PROTONS OF HEROIN 0.1 MOL dm^{-3} IN PHYSIOLOGIC DEUTERATED BUFFER AT pH = 6.8 AND T = 310°K

Resonance	R^{nS}	R^{S}	R^{nS}/R^{S}
H_1	0.67 ± 0.02	0.70 ± 0.03	0.96 ± 0.07
H_2	0.86 ± 0.02	0.82 ± 0.03	1.05 ± 0.06
H_7	0.98 ± 0.04	0.93 ± 0.05	1.05 ± 0.11
H_8	1.30 ± 0.05	1.24 ± 0.06	1.05 ± 0.09
H_5	1.28 ± 0.04	1.17 ± 0.04	1.09 ± 0.08

rapidly exchanging with the bulk to some receptors located either in the whole blood or in the plasma.

It must be recognized, however, that in our conditions it was not possible to observe heroin hydrolysis contrarily to the several reports of its occurrence in vitro (6–9). Since the number of heroin molecules was overwhelmingly more abundant than that of receptor sites, as compared with conditions where the hydrolytic reaction was observed, it is likely that the few hydrolyzed molecules escaped NMR observation.

Details of the binding interaction between heroin and the receptor site could be gained in the following way. From Eq. 1, when $p_b \ll 1$ and, hence, $p_f \simeq 1$ (which is our case) the following equation can be derived:

$$\Delta R^{S} = R^{S}_{obs} - R^{S}_{blank} = p_b R^{S}_{B}. \tag{6}$$

The reaction equilibrium with the receptor site (RCP) is schematized as

$$H + RCP \rightleftharpoons RCP - H; \tag{7}$$

the apparent equilibrium constant is given by

$$K = \frac{[RCP - H]}{[H]\,[RCP]} = \frac{[RCP - H]}{[H]\,\{[RCP]_0 - [RCP - H]\}}, \tag{8}$$

where $[RCP]_0$ represents the initial concentration of receptor sites.

The fraction of bound heroin molecules p_b is given by

$$p_b = \frac{[RCP - H]}{[H] + [RCP - H]} \simeq \frac{[RCP - H]}{[H]}. \tag{9}$$

From Eq. 8 the following rearrangement is possible:

$$K\,[H]\,\{[RCP]_0 - [RCP - H]\} = [RCP - H]$$
$$\times K\,[H][RCP]_0 - K[H][RCP - H] = [RCP - H]$$
$$\times K[H][RCP]_0 = [RCP - H]\{1 + K[H]\}$$
$$[RCP - H] = \frac{K[H][RCP]_0}{1 + K[H]}. \tag{10}$$

Substituting Eq. 10 into Eq. 9 and Eq. 6 yields

$$\frac{1}{\Delta R^{S}} = \left(\frac{1}{K} + [H]\right)\frac{1}{R^{S}_{b}\,[RCP]_0}. \tag{11}$$

TABLE II
ΔR^{S} VALUES FOR SELECTED LOW FIELD HEROIN PROTONS UPON ADDITION OF BLOOD FRACTIONS

Sample*	H_1	H_7	H_5
blood	0.20	0.22	0.21
Red blood cells 4%	—	—	—
Red blood cells 46%	0.04	0.07	0.02
serum	0.08	0.07	0.11
plasma	0.40	0.69	0.23

*100 μl of different preparations were added to 500 μl of heroin 0.1 mol dm^{-3} in D_2O at pH 6.8 and T = 310°K.

It is consequent that extrapolation of the plot of $1/\Delta R^{S}$ against $[H]$ to $1/\Delta R^{S} = 0$ allows evaluation of the apparent binding constant. The plot is shown in Fig. 3 and the extrapolation to $1/\Delta R^{S} = 0$ yields $K \simeq 39$ mol^{-1} dm^{-3}.

The value of K can be, moreover, used for describing the molecular dynamics of heroin at the receptor site. If binding to fibrinogen is assumed on the basis of absence of receptor sites in the human serum, and if the average concentration of fibrinogen in human plasma is considered (2–4 mg/ml), the R^{S}_{b} value of bound heroin molecules can be evaluated from Eq. 11.

Eqs. 3 and 4 give a certain R^{S} value as a function of $f(\tau_c)/r^6$, from which the correlation time can be calculated if r is known or vice versa. By looking at the molecule, it can be recognized, that the H_7 and H_8 protons are rather isolated from interactions with other ^1H dipoles such that the relaxation pathway can be suggested to consist exclusively of mutual H_7–H_8 dipole-dipole interactions. As a consequence τ_c of the bound heroin molecules could be calculated by assuming the $r_{7,8}$ distance of the solid state structure (13) ($\tau_c = 2 - 6 \times 10^{-6}$ s at 310°K). The calculated τ_c suggests a very tight binding interaction.

It should be recognized at this point, that similar calculations could be performed for other hypothetic receptor sites, provided the $[RCP]_0$ is known, but it must be concluded anyway that the receptor site for the tight binding of heroin is found in the plasma. It should also be stated that the reported τ_c for the bound heroin molecules represents an upper limit since it was calculated under the assumption that the H_7–H_8 dipole-dipole interaction is the only relaxation mechanism. Other H-H dipole-dipole interactions, either intramolecular or intermolecular with protons located at the receptor site, would yield lower values for $\rho_{7,8}$ and $\sigma_{7,8}$ and, hence, shorter values of the motional correlation time.

Returning to the problem of hydrolysis, if it occurs, as it should, it is not expected to lead to serious errors in the understanding of heroin's binding features.

In fact one should not forget that the NMR parameters are detected in the bulk, where memory of the bound environment is still retained, and that the measured R values are averaged over all the fast-exchanging heroin molecules in whichever environment.

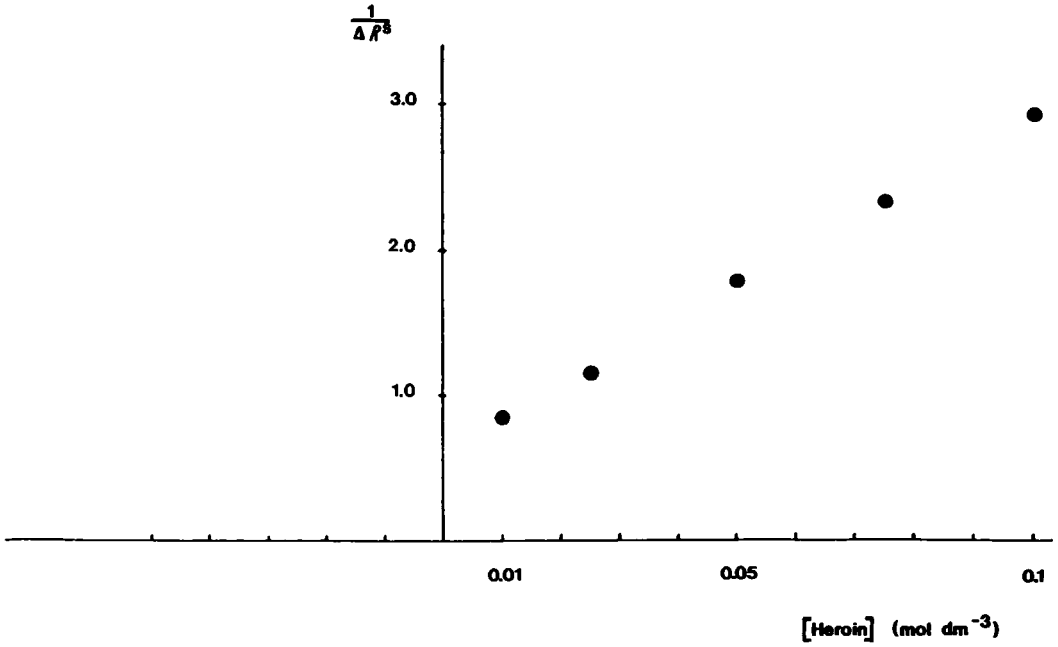

FIGURE 3 ($1/\Delta R^S$ (H_8 proton) vs. [heroin]) plot in the presence of human plasma (200 μl of plasma added to 500 μl heroin solution in physiologic deuterated buffer). pH 6.8 and T = 310°K.

Monoacetylmorphine or morphine molecules have different chemical shifts and, hence, are not expected to contribute to the observed relaxation behavior. Moreover, the extent of hydrolysis in our samples cannot exceed 1–2%, since, otherwise, the methyl groups of acetate could not have escaped NMR detection.

It can be concluded that measuring the selective proton spin-lattice relaxation rates of the low-field protons of the heroin molecule in physiologic conditions allows the following considerations. (*a*) Heroin undergoes a binding interaction with some receptor site located in the whole human blood or in the human plasma. (*b*) The binding brings about enhancement of the selective spin-lattice relaxation rates of protons located in different parts of the heroin molecule, such that the binding site cannot be unequivocally determined. (*c*) The apparent binding constant can be quoted at K = 39 mol^{-1} dm^3 from extrapolation of the $1/\Delta R^S$ vs. [H] plot. (*d*) A tentative assignment of fibrinogen as the receptor-carrying macromolecule allows delineation of the motional features of heroin molecules in the bound environment.

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