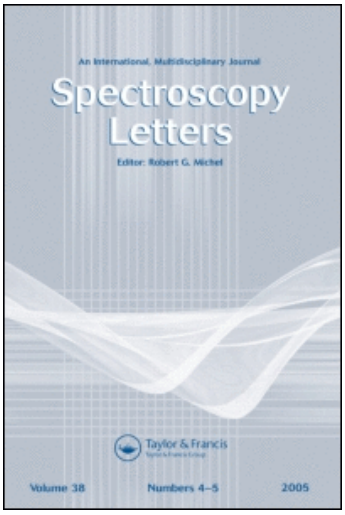

Spectroscopy Letters
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597299

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Online publication date: 31 October 2001

To cite this Article Coletta, Flaviano(2001) 'A STUDY OF WATER T_1 AND T_2 NMR RELAXATION TIMES IN HEALTHY AND CANCER AFFECTED HUMAN BLOOD PLASMA DOPED WITH HEMATOPORPHYRIN IX DYE', Spectroscopy Letters, 34: 5, 579 — 589
To link to this Article: DOI: 10.1081/SL-100106872
URL: http://dx.doi.org/10.1081/SL-100106872

SPECTROSCOPY LETTERS, 34(5), 579–589 (2001)

A STUDY OF WATER T_1 AND T_2 NMR RELAXATION TIMES IN HEALTHY AND CANCER AFFECTED HUMAN BLOOD PLASMA DOPED WITH HEMATOPORPHYRIN IX DYE

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ABSTRACT

Human blood plasma water protons relaxation times T_1^b and T_2^b in the protein bound water phase restricted motion state have been determined for the native and hematoporphyrin IX (**HMP**) doped samples at the temperature of 20 °C and ^{1}H NMR frequency of 80 Mhz. The samples belong to either healthy and myeloma affected patients. The resulting bound water relaxation times show a clear dependence from a single irrotational correlation time τ_c^b, related to the relatively slow motion of the proteins present in solution. Noteworthy the values of the τ_c^bs slow down in presence of the **HMP** dye and the effect is more marked in the myeloma blood plasma than in the healthy samples. This observation agrees with the measurements done previously with spectrofluorimetric techniques which show a specific interaction, time persistent, between the **HMP** and the fraction of the lipoproteins in

plasma. The rationalization of the experimental data using the homonuclear magnetic dipolar interaction equations of Solomon-Bloembergen enables to point out that one proton of the water molecule is relaxed by two nearby protons, one of which belongs to one of the hydroxyethyl side chains of the **HMP** linked with a hydrogen bond to the probed water molecule and stable on the NMR timescale.

Key Words: Blood plasma; Water T_1 and T_2 relaxation times; Healthy and myeloma affected patients; Hematoporphyrin IX effect

INTRODUCTION

The set of the molecules containing the cyclic tetrapyrrole porphyne ring constitutes one of the most interesting molecular classes in the chemical sciences. Their properties cover several chemistry frontier fields which go from the inorganic catalysis in oxidative processes[1,2] up to fundamental biologic activity, *i.e.* the photosynthesis[3] and the oxygen molecule capture and transport in the blood circulation[4]. One of the latest application of their photophysical properties[5,6] is the Photo Dynamic Therapy (**PDT**) of some types of cancer, whose mechanism is discussed in detail in many recent papers[7,8]. In this last context the overwhelming role of the blood plasma lipoproteins fraction in the capture before and the successive cells delivery of the **HMP** has been evidenced with spectrofluorimetric methods[9,10].

In this paper, prompted by a lack of papers which deal with NMR measures in blood plasma water doped with **HMP**, the ^1H nucleus relaxation times T_1 and T_2 have been measured for the pure water in the SPT state, and in the native blood plasma belonging to healthy and myeloma tumour affected people. Successively all the previous samples have been treated with variable amounts of **HMP** and the water protons relaxation times have been determined in a second set of experiments. The analysis of the experimental data allows us to retrieve the plasma protein bound water T_1^b and T_2^b relaxation times. These parameters have been interpreted with the use of the Solomon-Bloembergen magnetic dipolar interaction equations[11]. The results of this approach evidences how in all the samples with and without **HMP** dye a single rotational correlation time τ_c^b characterizes both the bound water relaxation times. The values of the τ_c^b decrease slightly from the healthy to the myeloma affected patients according to the well documented increase of both water relaxation times in tumoral tissues[12–14]. In the samples doped with **HMP** the bound water correlation times τ_c^b follows approximately the

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content of the lipoprotein fraction in the blood plasma, which in average is greater in the myeloma bearing than in the healthy people samples. In the author's opinion this fact can be assumed as an indication of the specific interaction between the **HMP** dye and the lipoproteins[9,10]. A further consequence of this study, supported by the data obtained for the system: water/**HMP**, with extension to the healthy and myeloma affected blood plasma water samples also containing **HMP**, is the fact that a single proton in one water molecule seems to be relaxed by two quasi nearby protons one of which is arranged in a hydrogen bonded structure resembling

$$[\mathbf{H_2O}\cdots\mathbf{HO} - \underset{\displaystyle \mathbf{CH_3}}{\underset{|}{\mathbf{CH}}}-]$$

with one of the hydroxyethyl side chains of the **HMP** dye.

EXPERIMENTAL SECTION

HMP purchased from Aldrich is purified according to the procedure described by Vever Bizet et al.[6] and the purity, checked with HPLC, is about 99%. A stock solution of $10^{-2}\,ML^{-1}$, corresponding approximately to 6 mg/ml which amounts to the dose per Kg of body weight normally used in the Photo Dynamic Treatment of the irradiable tumours[9,10] with visible light, is prepared in SPT water containing 10^{-2} ML^{-1} phosphate buffer[6] and the solution pH is adjusted to reach the physiological one. The stock solution is stored at 4 °C under nitrogen atmosphere in an amberized cruet in order to minimize as much as possible decomposition reactions induced by light.

The healthy and myeloma affected blood plasma samples are obtained as described in a previous work[15], all delivered with their respective clinical analysis data sheets with particular emphasys on the total proteins content composition. The water protons relaxation times T_1 and T_2 are determined respectively with the Inversion Recovery and the Hahn method as modified by Carr, Purcell, Meiboom, Gill[11,16] with the use of suitable microprograms written for the Bruker WP 80 SY NMR FT instrument at the ^{1}H nominal frequency of 80 Mhz. The experimental work instrumental parameters are: SW of 240 Hz for the water protons resonance peak, DR of $6\,10^{-2}$ Hz/Pn, maximum RD of 15 s, NS of 10 due to the good S/N ratio of the water resonance peak, ^{1}H π pulse width of 9.4 μs and variable delay time τ list which cover the range 0.1–6.0 s for the T_1 measures and $2.0\,10^{-3}$–2.05 s for the T_2 determinations. For each relaxation decay a minimum of 40 time data points are collected.

The samples studied in this work are in succession:

- ***a*** Pure water buffered at physiological pH around 7.0.
- ***b*** Healthy patients blood plasma (10 different samples) in the native state and then diluted with the sample ***a*** to achieve a concentration range, expressed as $\mathbf{c} = \mathbf{g}$ of total proteins per g of solution[14], variable between $7.5\,10^{-2}$ and zero (pure water).
- ***c*** The same as ***b*** but applied to the myeloma affected patients blood plasma (10 different samples) in which **c**, defined in ***b***, ranges between $9.0\,10^{-2}$ and zero.
- ***d*** **HMP** solutions in bufferized water with $\mathbf{c_{HMP}}$ in the interval between $5\,10^{-5}$–$10^{-2}\,\mathrm{ML}^{-1}$, step $\Delta\mathbf{c_{HMP}} = 4.3\,10^{-4}\,\mathrm{ML}^{-1}$ and including the pure water as the zero **HMP** concentration point.
- ***e*** Sample ***b*** with addition of variable amounts of **HMP** in the range from 0.0 up to $10^{-2}\,\mathrm{ML}^{-1}$, step $\Delta\mathbf{c_{HMP}} = 5.0\,10^{-5}\,\mathrm{ML}^{-1}$ with **c**, defined in the sample ***b***, variable between $7.5\ 10^{-2}$ and $6.7\ 10^{-2}$.
- ***f*** Sample ***c*** treated as ***e***.

All the samples are prepared at 4 °C in 3 mm. o.d. capillary tubes which are then adapted with Teflon spacers to amberized Trimital 5 mm. o.d. NMR tubes, with the external space filled with CD_3OD, 99,9% in Deuterium nuclei, for the magnetic field/frequency spectrometer lock. The measures are done at the probe temperature of 20 ± 1 °C with the built in the probe temperature controller B-VT 1000. For every sample a least of three time decay determinations are performed at random time intervals providing that in the dead times the samples are always stored at 4 °C at the dark. All the experimental time decay data points are processed with a non linear least square fit program from which the best water protons average relaxation time $< T_1 >$ and $< T_2 >$ are derived for each sample with the interval of confidence assessed between the 90 and 95%, depending from the actual experiment. I have got sufficiently good linear plots of the relaxation times vs **c** and $\mathbf{c_{HMP}}$ as discussed forward in the paper.

RESULTS AND DISCUSSION

With the obvious exception of pure water buffered at physiological pH, the plots of all the other blood plasma solutions proton water average $< T_1^{-1} >$ and $< T_2^{-1} >$ relaxation times vs the substrate concentration change either in the native and in **HMP** doped samples follow the general linear relationship

$$\mathbf{y_i} = \mathbf{A_i} + \mathbf{B_i x_j} \tag{1}$$

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with the index $\mathbf{i} = 1, 2$, $\mathbf{y_i} = \langle T_i{}^{-1} \rangle_{Exp}$, $\mathbf{A_i} = T_i^{f-1}$, $\mathbf{B_i}$ is equal[17] to $\left[\frac{T_i^{f-1} - T_i^{b-1}}{[H_2O]}\right]$ for $\mathbf{x_j} = \mathbf{c_{HMP}}$ and assumes the value $\mathbf{w}\left[\frac{1}{T_i^b} - \frac{1}{T_i^f}\right]$ for $\mathbf{x_j} = \mathbf{c}$[14] as defined before. In the previous expressions the indexes **b** and **f** refer to the bound and the free phase of water protons and **w** is stated as [g of water bound/g of dry total proteins][14].

The linear relationship as Eq. (1) is easily derived on the assumption of water protons two sites exchange[14–16] for all the samples studied in this paper and with the condition that the **HMP** does not forms intermolecular stacked aggregates in solution, as observed with fluorescence decays experiments at low **HMP** concentrations[6]. In this circumstance the hydration complex concentration $[\mathbf{HMP \cdots H_2O}]$ is equal to the initial concentration $[\mathbf{HMP}]_0$[17] providing that $[\mathbf{HMP}]_0 \ll [\mathbf{H_2O}] = 55.6\,ML^{-1}$ as happens in the reported experiments. The Table 1 lists the relevant linear coefficients $\mathbf{A_i}$ and $\mathbf{B_i}$, the linear corrrelation parameters $\mathbf{R_i}$ and the bound water protons relaxation times $\mathbf{T_i^b}$. The SPT pure water protons relaxation times are also reported in the Table 1 for comparison. These last values would be equal in the limit of very fast correlation time[15,16] and this assertion is correct for absolutely pure and accurately degassed water samples. The difference found between the two water proton relaxation times can be attributed to the paramagnetic effect of the dissolved molecular oxygen as discussed

Table 1. Average Linear Regression Coefficients $\mathbf{A_i}^{\$}$, $\mathbf{B_i}^{\$}$ of the Water Protons Relaxation Rates $< \mathbf{T_i}^{-1\$} >$ in Different Physical Surroundings and Irrotationally Bound Water Protons Relaxation Times $\mathbf{T_i^{b\S}}$ at 20 °C and 80 Mhz

Sample	$\mathbf{A_1}$ (s^{-1})	$\mathbf{B_1}$ ($s^{-1}C^{\#}$)	R_1	$\mathbf{A_2}$ (s^{-1})	$\mathbf{B_2}$ ($s^{-1}C^{\#}$)	R_2	$\mathbf{T_1^b}$ (s)	$\mathbf{T_2^b}$ (s)
a							2.93	1.40
b*	0.353	3.00	0.95	1.01	66.4	0.85	$9\,10^{-2}$	$4\,10^{-3}$
c*	0.360	2.40	0.91	1.02	43.0	0.87	$11\,10^{-2}$	$7\,10^{-3}$
d	0.350	1.65	0.97	0.90	2.9	0.97	$1.1\,10^{-2}$	$6.2\,10^{-3}$
e*	0.500	0.25	0.98	4.30	13.72	0.80	$7\,10^{-2}$	$1.3\,10^{-3}$
f*	0.590	0.20	0.81	4.32	16.40	0.84	$8.4\,10^{-2}$	$1.1\,10^{-3}$

Notes: $^{\$}$ A_i, B_i with $\mathbf{i} = 1, 2$ are the coefficients of the linear plots of $< T_i^{-1} >$ vs **c** and $\mathbf{c_{HMP}}$.

* Average values over 10 different samples.

§ With the exception of the pure water all other protons relaxation times refer to the substrate bound water phase.

$^{\#}$ C is a substrate concentration parameter which assumes the values of **c** and $\mathbf{c_{HMP}}$ (refer to the Experimental section).

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thoroughly successively in this paper. The blood plasma proteins bound water proton relaxation times confirm the well known significative increase of the two parameters in blood plasma water of cancer affected samples compared to healthy ones[12,13,15].

The blood plasma water protons relaxation times, in samples with an initial average total proteins contents of $7.5\,10^{-2}$ g/g of solution, when diluted with water, give the slope of $3.0\,s^{-1}$ C for $< T_1^{-1} >$ and $66.33\,s^{-1}$ C for $< T_2^{-1} >$. The values of the slopes obtained by Daszkiewicz[14] in a dilution study of ovoalbumin are $3.07\,s^{-1}$ C and $6.8\,s^{-1}$ C at 20 °C and 14 Mhz. At this last NMR proton resonance frequency the bound water protons $\mathbf{T_1^b}$ and $\mathbf{T_2^b}$ are 4.4 ms and 2.2 ms with the protein hydration water parameter **w** equal $1.6\,10^{-2}$ g of bound water/g of dry protein. From the experimental data reported in the Table 1 the $\mathbf{T_1^b}$ values are 90 ms and 110 ms and 4 and 7 ms for $\mathbf{T_2^b}$[15,19,20] for healthy and myeloma affected samples. The parameter **w** equals 0.31[19], the irrotational correlation time τ_c^b results $1.1\,10^{-8}$ s in agreement with that reported in the reference 14. The strong discrepancy of the parameter **w** can be surely ascribed to the fact that at high magnetic field the assumptions done by Daszkiewicz[14] for the validity of the water two sites fast exchange condition extend further in water shell regions more distant from the immediate protein binding centers. This evidence follows directly from the increase of both $\mathbf{T_1^b}$ and $\mathbf{T_2^b}$ with the intensity of the magnetic field[13]. The further decrease of the irrotationally bound water protons relaxation times in pure SPT water, in healthy and cancer affected blood plasma doped with **HMP** seems interesting.

A simple calculation, using the Debye-Einstein equation[16] and based on the geometry of the **HMP** molecule including the hydroxyethyl side chains and treated like a whole rigid top, with the **HMP** hindrance radius of about 15 A, gives the isotropic rotational correlation time τ_c equal to $3.1\,10^{-9}$ s. Inserting this τ_c in the full frequency dependent Bloembergen-Solomon equations[11,16], $\mathbf{T_1^b}$ and $\mathbf{T_2^b}$ values of $3.2\,10^{-2}$ s and $6.2\,10^{-3}$ s are obtained for the complex [$\mathbf{HMP\cdots H_2O}$], with the water molecule assumed firmly bound to the **HMP** substrate, which are of the correct order of magnitude of the experimental data shown in the Table 1. The same considerations seem to apply to the blood plasma water protons, doped with the **HMP** dye, in healthy and cancer affected samples with $\mathbf{T_1^b}$ values intermediate between that of the undoped samples and **HMP** doped pure water whereas the $\mathbf{T_2^b}$ values fall on the shorter side.

The Table 2 reports the water protons irrotational correlation times τ_c^b for the different samples. For sake of comparison also the rotational correlation time τ_c of the pure water protons are quoted. In the same table are listed the experimental and calculated bound water protons relaxation rates. The full homonuclear magnetic dipolar relaxation equations[16] are

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Table 2. Irrotational Correlation Times τ_c^b, Experimental and Calculated Bound Water Protons Relaxation Rates in Different Physical Surroundings at 20 °C and 80 Mhz

Sample	$\tau_c^b 10^8$ (s)	$T_1^{b-1}{}_{exp}$ (s^{-1})	$T_2^{b-1}{}_{exp}$ (s^{-1})	$T_1^{b-1}{}_{calc}$ (s^{-1})	$T_2^{b-1}{}_{calc}$ (s^{-1})
a	$2.3\,10^{-4}$	0.342	0.342	0.342	0.342
b*	1.08	11.30	247	11.11	243
c*	0.98	9.10	143	12.14	221
d	0.20	90.10	162	80.10§	177§
e*	1.70	14.30	769	14.11§	765§
f*	2.01	11.90	900	12.01§	900§

Notes: * Average values over 10 different samples.
§ Values calculated assuming the number of vicinal relaxing protons $\mathbf{N_H} = 2$.

$$\frac{\mathbf{1}}{\mathbf{T_1^b}} = \mathbf{A}\left\{\frac{\tau_c^b}{1+\omega^2\tau_c^{b2}} + \frac{4\tau_c^b}{1+4\varpi^2\tau_c^{b2}}\right\}\mathbf{N_H}, \quad \text{and} \tag{2}$$

$$\frac{\mathbf{1}}{\mathbf{T_2^b}} = \mathbf{B}\left\{3\tau_c^b + \frac{5\tau_c^b}{1+\varpi^2\tau_c^{b2}} + \frac{2\tau_c^b}{1+4\varpi^2\tau_c^{b2}}\right\}\mathbf{N_H} \tag{3}$$

where $A = 2B = \frac{3}{10}\frac{\gamma^4\hbar^2}{r^6_{H\cdots H}} = 1.5\cdot 10^{10}\,s^{-2}$, $\mathbf{N_H}$ is the number of the nearby relaxing $^1\mathbf{H}$ nuclei, $\mathbf{r_{H\cdots H}}$ is the average internuclear distance and the other constants are self explaining. For the pure water, the condition $(\omega\tau_c)^2 \ll \mathbf{1}$ applies, whose consequence is that $\mathbf{T_1}$ is equal to $\mathbf{T_2}$[16,18]. When paramagnetic molecules are dissolved in water, the equality of the two relaxation times is no longer guaranteed as reported by several authors[11,16,18,21]. In SPT water the paramagnetic centers are the dissolved $\mathbf{O_2}$ molecules in the singlet state with the electron spins number $\mathbf{S} = \mathbf{1}$. The difference of the water protons relaxation rates under the magnetic interaction with the electron spins, assuming negligible the proton–electron hyperfine constant **A**, is then given[21] by the expression

$$\frac{\mathbf{1}}{\mathbf{T_2}} - \frac{\mathbf{1}}{\mathbf{T_1}} = \frac{\mathbf{1}}{\mathbf{15}}\mathbf{S(S+1)}g_e^2\beta_e^2\frac{\gamma_H^2}{r_{en}^6}\left\{\tau_c - \frac{\tau_c}{1+\varpi_s^2\tau_s^2}\right\}\frac{\mathbf{N_{O_2}n}}{\mathbf{N_P}} \tag{4}$$

where $[\mathbf{S(S+1)}]^{\mathbf{1/2}}\mathbf{g_e}\beta_e = 1.2$ Bohr magnetons[18], $\omega_s = 3.3\,10^{11}\,rad\,s^{-1}$ is the electron spin resonance frequency at the magnetic field intensity of 1.879 T, τ_s is the electron spin correlation time, $\tau_c = 60.3\cdot 10^{-12}\,s$ is the rotational

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correlation time calculated for the complex $[\mathbf{H_2O\cdots O_2\cdots H_2O}]$ with $\mathbf{N_{O^2}} = 1$, $\mathbf{n} = 2$ and $\mathbf{N_P} = 4$ and molecular radius $\mathbf{r_{en}} = 4.1$ A. Inserting the appropriate numeric values for all the variables in the Eq. (4), the difference between the water relaxation rates results $0.47\,s^{-1}$ which compares well with the experimental value of $0.38\,s^{-1}$ derived from the data reported in the Table 1. The irrotational correlation times τ_c^b of the blood plasma water protons firmly bound to the proteins in both healthy and cancer affected samples listed in the Table 2 are obtained with a reasonable simplification of the full Eqs. (2) and (3) based on the results of several experiments[14,15,19,20].

Under the condition $(\omega\tau_c^b)^2 \gg 1$, the Eqs. (2) and (3) reduce to the simpler form

$$\frac{1}{T_1^b} \cong 2A\left\{\frac{1}{\omega^2\tau_c^b}\right\}N_H, \quad \text{and} \tag{5}$$

$$\frac{1}{T_2^b} \cong B\{3\tau_c^b\}N_H \tag{6}$$

where in the Eq. (6) the frequency dependent contributions of the random fluctuating nuclear magnetic fields are neglected compared to the magnitude of the secular static term. From the values of T_1^b and T_2^b reported in the Table 1 for the blood plasma water protons result $\tau_c^b = 1.08\,10^{-8}$ s in the healthy and $\tau_c^b = 0.98\,10^{-8}$ s for the cancer affected samples. The τ_c^b for the water protons relaxation times, when the **HMP** alone is present, are derived from Eqs. (2) and (3) under the suitable approximation $(\omega\tau_c^b)^2 \cong 1$. These approximate water protons correlation times are used in the full relaxation Eqs. (2) and (3) to give the $T_1^{b-1}{}_{calc}$ and $T_2^{b-1}{}_{calc}$ listed in the Table 2 which compare well with the experimental data. It is worthy to recall that the good agreement between the two sets of relaxation rates, for all the samples containing **HMP**, is reached only if in the Eqs. (2) and (3) the number of nearby relaxing protons N_H around one of the water proton is set to 2. This fact suggests that only one molecule of water can cohordinate, via a stable hydrogen bond, to the **HMP** molecule. The **HMP** molecular fragment candidate to form stable hydrogen bonds is the hydroxyethyl side chain. One further consideration comes out when a comparison of the **HMP** doped blood plasma water protons correlation times τ_c^b is done in both healthy and myeloma bearing samples. The ratio between the two quantities, 0.85, approximates the average ratio, 0.77, of the total lipoproteins present in the blood plasma, as derived from the blood clinical analysis sheets, 0.72 g/100 g of solution for the healthy and 0.95 g/100 g of solution for the myeloma

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affected patients. This confirms, with the use of a totally different experimental technique, what has been previously detected by using fluorescence decays methods[9,10]. The results of these experiments show clearly that the water protons mobility of the complex [**HMP** $\cdots$ $\mathbf{H_2O}$], measured by the relative values τ_c^b, is lower in the myeloma affected samples and support the existence of a specific affinity between the previous complex and the lipoproteins fraction a little bit higher than in the healthy subjects. This fact has been exploited in the search of chemical modifications in the porphyrin nucleus[7–10] with the aim to increase the selective absorption effects between healthy and cancer affected tissues for a more efficient **PDT** treatment of the tumours.

ACKNOWLEDGMENTS

I thank the Director of the CSSMRE, Prof. Giovanni Giacometti, for his interest about this work, Dr. Alberto Tregnaghi for the samples gift, Prof. Giulio Jori of the Biology Department for introducting myself in the **PDT** field and the Italian C.N.R. for the financial support.

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Received January 25, 2000
Accepted July 1, 2001

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