Albumin-bound MRI contrast agents: the dilemma of the rotational correlation time

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Abstract

Human serum albumin (HSA) binds numerous molecules, among which are suitably designed MRI contrast agents. The rotational tumbling of the protein is thus one of the parameters likely to affect the in vivo relaxivity of these agents. Literature unveils discrepancies about the value of the rotational correlation time (τ_R) of HSA. In the present work, the τ_R of this protein has been determined by studying the deuterium relaxation rate of small molecules known for their strong binding to HSA (warfarin and 4-hydroxycoumarin). Values of approx. 20–22 ns are obtained at 310 K in a 4% HSA solution and are in good agreement with the theoretical predictions. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

The vascular blood lifetime of small gadolinium complexes used as MRI contrast agents can be prolonged by non-covalent interaction with albumin. Such a binding also results in a reduction of molecular mobility and, in most cases, leads to an enhancement of proton relaxivity [1].

The non-covalently bound Gd-complexes studied so far, however, seem to tumble faster than the protein [2–4]. This may be indicative of some freedom of the Gd–water moeity, but quantitative interpretations are difficult because the human serum albumin correlation time (τ_R) values reported in the literature are divergent [5–12] (Table 1).

In this work, we determine the rotational correlation time of human serum albumin (HSA) by deuterium relaxometry of specifically labeled probes known for their high affinity for the protein. Deuterium is chosen as a reporter because, in diamagnetic molecules, the relaxation rate of this nucleus predominantly arises from quadrupolar interactions modulated by the rotational motion [13]. Experimental values of τ_R will then be compared to those predicted by current theories and models.

2. Materials and methods

2.1. NMR spectrometry

All spectra (H-1, H-2, C-13) were recorded at 7.05 T on a Bruker AMX-300 instrument (Karlsruhe, Germany). For H-1 and C-13, 0.6 ml solutions were contained in 5 mm o.d. tubes; while for the H-2 analysis, 2 ml solutions were contained in 10 mm od. tubes (SW = 1 kHz, TR = 110 ms, $\pi/2$ pulse, SI = 32 K).

2.2. Chemicals

HSA (Sigma, catalog number A-1653, Bornem, Belgium) was used without further purification. Warfarin (Sigma, catalog number A3430, Bornem, Belgium) and 4-hydroxycoumarin (Aldrich, catalog number H 2,380-5, Bornem, Belgium) were deuterated using reported procedures [14,15]. Deuteration of 4-hydroxycoumarin was nearly complete on carbons 6, 7 and 8 as confirmed by ^{1}H and ^{13}C NMR spectra (solvent DMSO-d_6) (Fig. 1).

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Table 1
τ_R values reported for HSA

Method	Conditions	τ_R ns	Ref.
^{23}Na NMR	[HSA] = 4.25%, T = 22 ± 2°C	16 ± 6	[7]
Fluorescence of Trp	$T = 25$°C	31	[5]
Fluorescence of Trp	$T = 30$°C	32	[8]
	$T = 44$°C	23.5	
Fluorescence of Trp	[HSA] = 0.01% $T = 20$°C	41	[9]
Fluorescence of Trp	$T = 20$°C	26	[6]
EPR	$T = 20$°C	18	[10]
Calculation	$T = 20$°C	26.5	[6]
	$T = 20$°C	50	[11]
	$T = 37$°C	14	[12]

^{1}H NMR, δ(ppm): 7.9 (1H, s); 7.15(0.2 H, s); 4.6 (1H, s); ^{2}H NMR, δ(ppm): 7.7–7.4 (3 ^{2}H, m); ^{13}C NMR, δ(ppm):174.6 (s); 164.8 (s); 154.5(s); 129.6(t); 124.2 (s); 123.6 (s); 121.3(t); 115.4–115.1(t + s); 85.2(s)

Warfarin was deuterated on aromatic carbons as well as on methyl and methylene carbons (Fig. 1).

^{1}H NMR, D$_2$O, δ(ppm): 7.8(1 H, s); 7.3(2 H, s); 4.8(1 H, s);3.3 (0.3 H, d); ^{2}H NMR, D$_2$O, δ(ppm): 7.4 (6 ^{2}H, m); 3.4 (1.7 ^{2}H, s); 2.2 (3 ^{2}H, s); ^{13}C NMR, acetone-d_6, δ(ppm): 211.3(s); 173.9(s); 165.8(s); 155(s); 147.9(s); 129.7(t); 128.7 (s); 127.7 (t), 126 (s); 125.3 (t); 124.8 (s); 121.7 (t); 115.7 (t); 100.3 (s); 46.8 (m); 36.8 (s); the methyl resonance was not detectable due to the overlapping of acetone-d_6 peaks.

The concentrations of the stock solutions of deuterated compounds were obtained by comparison of the area of their ^{2}H resonances with a reference contained in a concentric tube. For warfarin, the referent was nitromethane-d_3 or D$_2$O and the dosage was based on the area of the methyl peak which was totally deuterated, as shown by the proton NMR spectra. For 4-hydroxycoumarin, the referent was D$_2$O and it was assumed that the aromatic lines represented three deuterium atoms.

2.3. Data treatment

The deuterium spectra were deconvoluted using the Linesim Bruker program. If the exchange between

bound and free ligands is fast as compared to the relaxation rates in the bound and free states (and provided the chemical shift differences between these two states is negligible), the transverse relaxation rate of the bound fraction (R_2^B) can be calculated from the linewidth ($\Delta v_{1/2}^{obs} = R^{obs/\pi}$), the transverse relaxation rate of the free ligand (R_2^F), and the molar fraction of free (X^F) and bound complexes (X^B), obtained from the known association constants (Eq. (1)).

$$R_2^B = \frac{R_2^{obs} - X^F R_2^F}{X^B}. \tag{1}$$

The rotational correlation time of the bound fraction is then calculated from Eq. (2)

$$R_2^B = \frac{3\pi^2}{20}\left(\frac{e^2qQ}{h}\right)^2\left[3\tau_R + \frac{5\tau_R}{1+\omega^2\tau_R^2} + \frac{2\tau_R}{1+4\omega^2\tau_R^2}\right] \tag{2}$$

where ω is the angular frequency of ^{2}H and e^2qQ/h, the quadrupolar coupling constant, is equal to 180 kHz (aromatic C–^{2}H bond).

3. Results and discussion

3.1. Theoretical predictions

By definition, a spherical molecule diffusing in a continuous medium rotates by ~ 33° over one rotational correlation time τ_R. τ_R is related to the diffusional rotational constant D_R which itself depends on the absolute temperature (T), the viscosity (η), the Boltzmann constant (k), and on the radius (a) of the molecule (Eq. (3) and Eq. (4)):

$$\tau_R = \frac{1}{6D_R} \tag{3}$$

$$D_R = \frac{kT}{8\pi\eta a^3}. \tag{4}$$

Combining Eq. (3) and Eq. (4) gives the well known Stokes–Einstein expression (Eq. (5)) relating the rotational correlation time to the molecular volume ($4\pi a^3/3$), the viscosity, and the temperature.

$$\tau_R = \frac{4\pi\eta a^3}{3kT}. \tag{5}$$

To account for the fact that the diffusing molecules do not move in a continuous medium, a microviscosity factor (f) as described by Gierer and Wirtz [16] has been introduced (Eq. (6)). The microviscosity factor depends on the ratio of the radius of the solvent (r_s) and of the solute (a) (Eq. (7)). When the solute molecules are much larger than those of the solvent, as for albumin in water, f tends to one and the Stokes–Einstein Eq. (5) can be used.
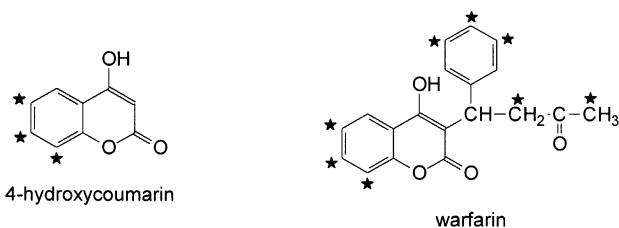

Fig. 1. Chemical structures of 4-hydroxycoumarin and warfarin. The labeling positions are shown by the stars.
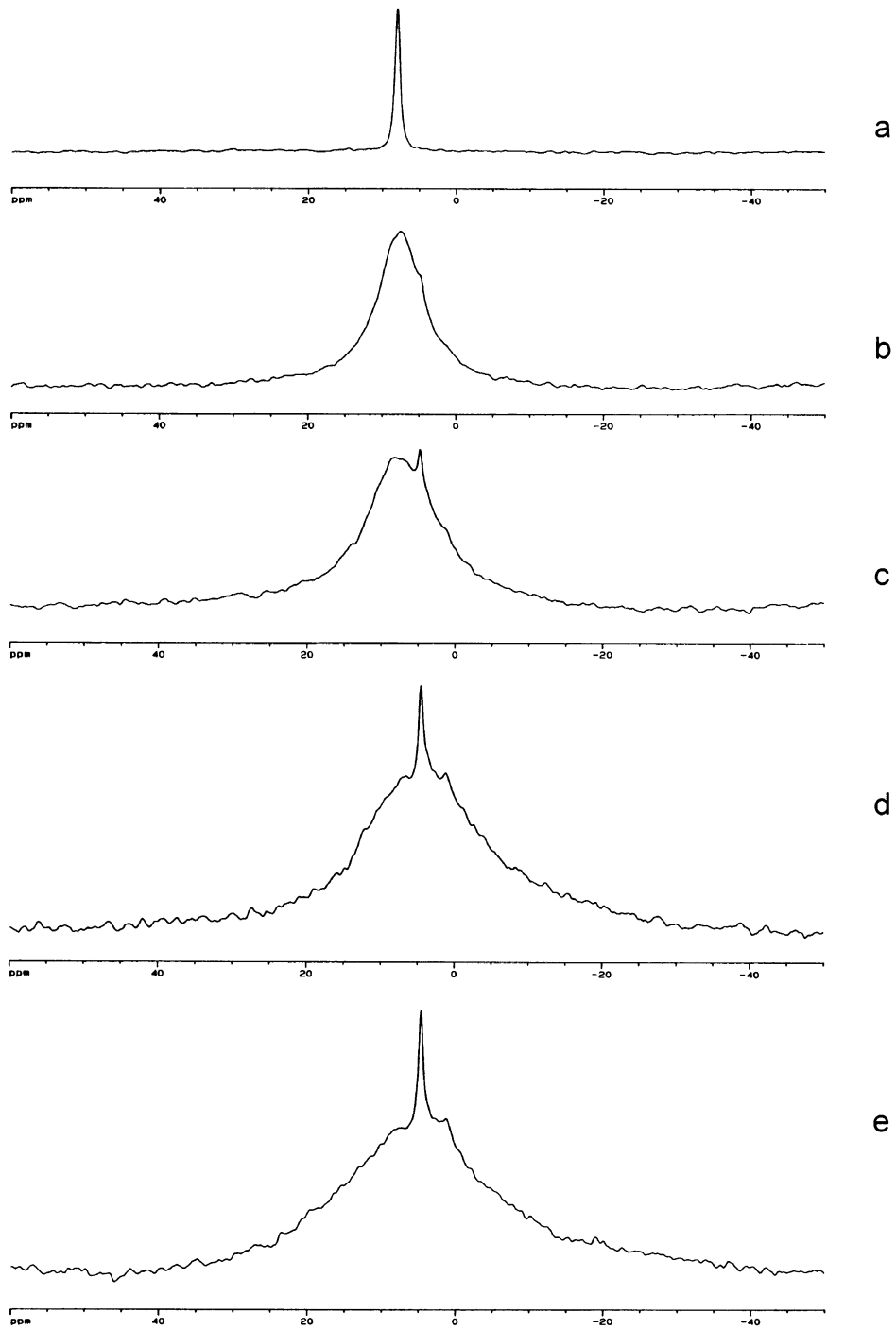
Fig. 2. Deuterium NMR spectra of 4-hydroxycoumarin-6,7,8-d_3: (a) 2.04 mM in water and (b–e) in 4% HSA solutions: 5.11 mM (b), 2.56 mM (c), 1.28 mM (d), 0.83 mM (e). The narrow line in spectra b,c,d, and e is arising from residual HDO.

$$\tau_R = f \frac{4\pi\eta a^3}{3kT} \tag{6}$$

$$f = \left[6\frac{r_s}{a} + \left(1 + \frac{r_s}{a} \right)^{-3} \right]^{-1}. \tag{7}$$

For proteins, a modified expression has been proposed [5,6] (Eq. (8))

$$\tau_R = \frac{M(v_s + h)\eta}{RT} \tag{8}$$

where M is the molecular weight of the protein, v_s is its specific volume (0.72–0.735 cm^3/g), h represents the degree of hydration (0.2–0.3 cm^3/g), η is the viscosity in poise, R is the gas constant, and T the absolute temper-
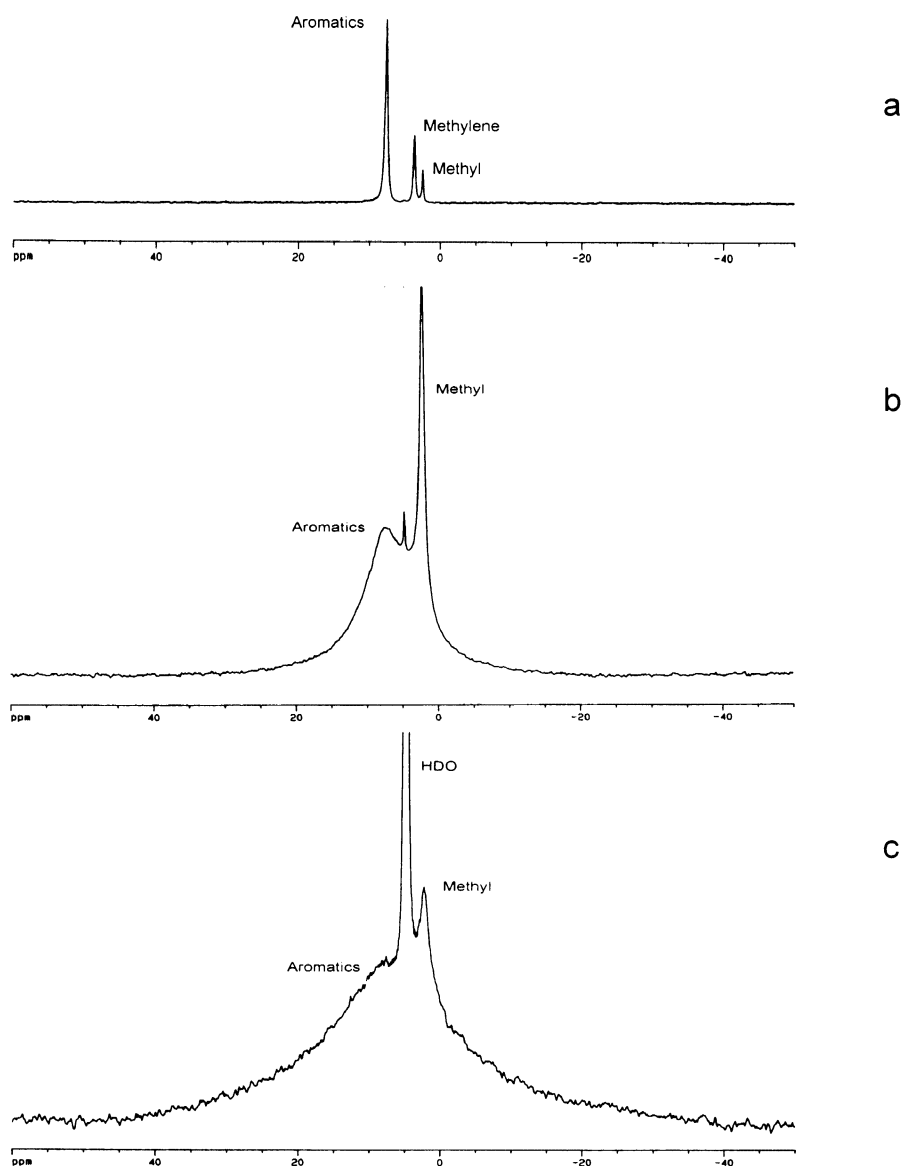
Fig. 3. Deuterium NMR spectra of deuterated warfarin: (a) 16.1 mM in water and (b–c) in 4% HSA solutions: 13.39 mM (b), 3.97 mM (c).

ature [5,6]. A value of 17 ns is obtained from the Stokes–Einstein equation for a 4% HSA solution (molecular volume 88248.9×10^{-30} m^3 [17], $\eta = 0.84$ cP at 310 K). If the microviscosity factor is taken into account, τ_R slightly drops to 15–16 ns. Larger values (20–22 ns) are obtained from Eq. (8).

3.2. Experimental study

The choice of the probes (4-hydroxycoumarin and warfarin; Fig. 1) was governed by their strong affinity for HSA and their structural rigidity. Indeed, the deuterated probes need to be tightly and closely bound to the macromolecule with as less individual mobility as possible in order to reflect the mobility of the host.

3.2.1. 4-Hydroxycoumarin

The deuterium spectra of deuterated 4-hydroxycoumarin in pure water and in the presence of HSA are
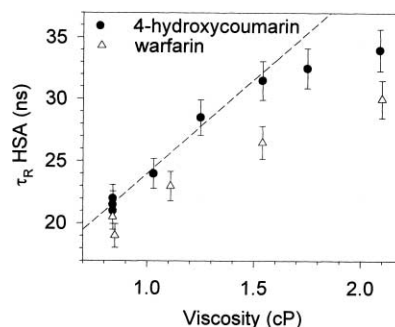

Fig. 4. τ_R versus viscosity of solutions containing 4–20% of HSA.

shown in Fig. 2. In aqueous solution free of protein, the two peaks at 7.4 and 7.7 ppm (see experimental section) are not visible because of the exponential multiplication ($LB = 30$ Hz) applied to the free induction decay. In the presence of HSA, a concentration dependent broadening of the deuterium resonances due to fast exchange between free and bound deuterated probes is observed. Zaton et al. reported that the interaction between 4-hydroxycoumarin and HSA is characterized by a strong binding site ($K_{a1} = 4.1 \times 10^5$ M^{-1}) and two weaker ones ($K_{a2} = 6.1 \times 10^3$ M^{-1}) [18]. Calculation shows, therefore, that in the solution containing 0.83 mM of 4-hydroxycoumarin and 4% of HSA, 95% of the probe is bound to the protein and predominantly (71%) to the high affinity site.

Deconvolution of the spectrum and subsequent use of Eq. (1) and Eq. (2) give a linewidth of 3320 Hz for the bound complex, corresponding to a τ_R of 22 ns. Two other samples containing 0.38 mM ($X_2^B = 99\%$) or 0.71 mM ($X_2^B = 97\%$) of 4-hydroxycoumarin were also analyzed. τ_R values of 21 and 22 ns were respectively obtained.

3.2.2. Waffarin

Similar results were obtained with deuterated warfarin. A concentration dependent broadening of the deuterium resonances was observed in HSA solutions (Fig. 3). The spectra are more complex due to the presence of two aliphatic resonances (C^2H_2 and C^2H_3). Because of the motion freedom of this aliphatic chain, the broadening of their signals is smaller than for the aromatic resonances. The determination of τ_R was thus based on the analysis of the aromatic resonance.

Three samples containing 1.18, 1.14 and 0.66 mM of deuterated warfarin and 4% of HSA were analyzed as described above. The bound fractions calculated from three references (two sites with $K_a = 7.1 \times 10^4$ M^{-1} and 1.73 sites with $K_a = 3.46 \times 10^3$ M^{-1} [18], two sites with $K_a = 1.5 \times 10^5$ M^{-1} and five sites with $K_a = 1.5 \times 10^3$ M^{-1} [19], one site with $K_a = 2.3 \times 10^5$ M^{-1} and four sites with $K_a = 5.9 \times 10^3$ M^{-1}[20]) are equal to 96–97, 96–97 and 98–99% respectively. From the R_2^B calculated values (3010–3115 Hz), a τ_R of 20–21 ns was obtained.

3.2.3. Influence of HSA concentration

HSA concentration is known to considerably affect the macroviscosity of the solutions and the Stokes–Einstein equation predicts that τ_R should increase linearly with the viscosity. The dependence of τ_R versus HSA concentration was thus investigated using the procedure described above and applied to solutions containing increasing amounts of the protein and concentrations of the deuterated ligands (4-hydroxycoumarin and warfarin) guaranteeing at least 95% of binding. The results presented in Fig. 4 nicely demonstrate a linear increase

of τ_R until η reaches 1.5 cP which corresponds to a HSA concentration of 16%.

The smaller values of τ_R obtained from the warfarin study can be related to the contribution of the more mobile deuterated phenyl cycle of the ligand to the overall aromatic resonance used for the analysis.

4. Conclusion

This study of the molecular tumbling of human serum albumin based on the analysis of the deuterium transverse relaxation rates of non-covalently bound deuterated ligands has shown that τ_R of albumin at 310 K in a 4% solution is about 20–22 ns and increases with HSA concentration. The values obtained are in very good agreement with the theoretical predictions, in spite of the fact that this analysis assumes an isotropic rotation and the absence of individual mobility of the reporters.

These values of 20–22 ns are therefore limits for the expected correlation time of a paramagnetic contrast agent non-covalently but tightly bound to HSA at 310 K in 4% protein solution. If the supramolecular assembly is somewhat flexible with regard to the paramagnetic moeity, the effective τ_R modulating the electron–nucleus interactions will consequently decrease and prevent the proton relaxivity to reach its maximum value.

Finally, it should be noted also that the determination of protein mobility through the analysis of deuterium relaxation rates of labeled reporters does not necessarily need a fast exchange between the free and the bound states. In the case of a slow exchange, however, a strong interaction between the protein and the ligand is needed in order to produce a large amount of bound material (> 80%) enabling the detection of its NMR spectrum [4].

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