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Synthesis of a new gadolinium complex with a high affinity for human serum albumin and its manifold physicochemical characterization by proton relaxation rate analysis, NMR diffusometry and electrospray mass spectrometry

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Abstract

A novel gadolinium complex, derived from Gd–DTPA (DTPA: diethylenetriaminepentaacetic acid) and sulfaphenazole, intended to be a potential MRI contrast agent and to interact with human serum albumin (HSA), was synthesized and characterized. Its relaxometric properties were evaluated in water, and its binding to HSA was investigated by three techniques: proton relaxation rate analysis, NMR diffusometry, and electrospray mass spectrometry. The complex has a higher relaxivity than the parent compound ($r_1 = 7.8\ s^{-1}\ mM^{-1}$ at 310 K and 0.47 T and $7.7\ s^{-1}\ mM^{-1}$ at 310 K and 1.41 T), a fast water exchange, and a very good stability versus zinc(II) transmetallation. All techniques agree with a high affinity of the complex for HSA, and competition experiments indicate that this contrast agent competes with ibuprofen for HSA.

Keywords: Contrast agent; Albumin; Interaction; NMR; Mass spectrometry

1. Introduction

Magnetic resonance imaging (MRI) is widely used as a clinical diagnostic tool thanks to its very good spatial and temporal resolution. Its main drawback is a poor sensitivity, which can be compensated by the use of contrast agents designed to enhance the nuclear relaxation processes of the water protons [1–6]. The first commercial MRI contrast agent was Magnevist®, a clinical formulation of Gd–DTPA (DTPA: diethylenetriaminepentaacetic acid, Schering, Germany) which is characterized by a biodistribution restricted to the vascular and extracellular spaces, and a rapid renal elimination. For early detection of some diseases, more specific contrast agents, able to recognize specific receptor molecules located inside or outside the cells, are needed. In addition, contrast agents with a high relaxivity are also necessary. For most of them, a higher relaxivity is achieved by reducing the mobility through an increase of the molecular size either by covalent or non-covalent binding to a large structure like a macromolecule. For example, several contrast agents able to bind to the endogenous serum albumin have been developed and have been shown to be very efficient [7–19].

Human serum albumin (HSA) is a 66 kDa blood protein, which is one of the most abundant proteins in plasma (concentration of about 4%) and which is known to bind to a large variety of ligands with a relatively high affinity. Two major binding sites can be distinguished, according to the Suddlow classification scheme [20–24]: the Suddlow site II, located in the subdomain IIIA of HSA, is a hydrophobic pocket and binds small aromatic carboxylic acids, like

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L-tryptophan, ibuprofen, or thyroxine, while the Suddlow site I, located in the subdomain IIA, is reported in the literature as "a large and flexible region" [23], and is able to bind a wide diversity of ligands among which are salicylate, warfarin or bilirubin.

In the present work, sulfaphenazole which has been reported to strongly bind to HSA or BSA (bovine serum albumin) (K_a of $7.5 \times 10^5\ M^{-1}$ ($n = 0.7$) [25], or ranging between 9.5×10^5 and $16.1 \times 10^5\ M^{-1}$ ($n = 0.5$–1) [26] for HSA at 298 K, and $1.55 \times 10^5\ M^{-1}$ ($n = 1$) [27] or $9.2 \times 10^4\ M^{-1}$ ($n = 1$) [28] for BSA at 310 K) was coupled to a derivative of Gd–DTPA to give Gd–C_4-sulfaphenazol-DTPA. This new C_4 substituted Gd-complex has a structure similar to that of MS-325 [7,8] and will be compared to this known compound throughout the study (Fig. 1).

The new complex was characterized *in vitro* in aqueous solution and in the presence of human serum albumin. Its interaction with HSA was evaluated by three different techniques: proton relaxation rate analysis [7–19], NMR diffusometry [29–39], and electrospray mass spectrometry [19,40]. The first method takes advantage of the relaxivity difference between the free paramagnetic complex and the supramolecular assembly resulting of its binding to HSA. Classically, the procedure consists of measuring the water proton relaxation rate of solutions containing increasing concentrations of gadolinium complex in a 4% aqueous solution of HSA. The experimental data can then be fitted with Eq. (1) in order to obtain an estimation of the association constant and of the number of binding sites, assuming that all sites are identical and independent.

$$R_{1\mathrm{obs}}^{\mathrm{P}} = 1000 \cdot \left\{ r_1^{\mathrm{l}} l^0 + \left(r_1^{\mathrm{c}} - r_1^{\mathrm{l}}\right) \left\{ \frac{np^0 + l^0 + K_a^{-1} - \sqrt{(np^0 + l^0 + K_a^{-1})^2 - 4nl^0p^0}}{2} \right\} \right\} \quad (1)$$

In this equation, p^0 and l^0 are the initial concentrations in HSA and in the gadolinium complex, respectively; r_1^{l} and r_1^{c} are the relaxivities of the free contrast agent and of the complex contrast agent/HSA, respectively; K_a is the association constant; and n is the number of binding sites.

The second technique used in this work is based on the analysis of the diffusion coefficient of the contrast agent in the bound and in the free states. The diffusion coefficient is measured by NMR in solutions containing various concentrations of the contrast agent and a fixed concentration of HSA. If the exchange between the contrast agent and the macromolecule is sufficiently rapid, the observed diffusion coefficient (D_{obs}) is the mean of the diffusion coefficient of the free contrast agent (D_f) and the diffusion coefficient of the bound contrast agent (D_b), weighted by the molar fraction of free (x_f) and bound contrast agent (x_b) (Eq. (2)) [32–39]

$$D_{\mathrm{obs}} = x_f D_f + x_b D_b \quad (2)$$

For this technique, it is necessary to replace the gadolinium ion by a poorly relaxing analogue in order to avoid an excessive reduction of the transverse relaxation time and a subsequent broadening of the NMR signals. Europium(III) ion was used since this lanthanide has the additional advantage of shifting the chelate signals far away from those of HSA, making it easier to measure the chelate's diffusion coefficient. One drawback of this NMR technique, however, is the impossibility to detect compounds with high affinity for HSA. Indeed, if the interaction is very

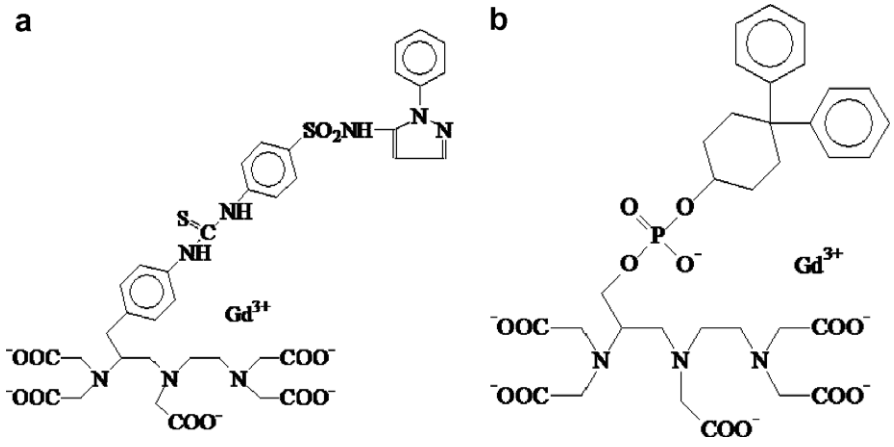

Fig. 1. Chemical structure of (a) Gd–C_4-sulfaphenazol-DTPA and (b) MS-325.

strong, the exchange is slow on the diffusion timescale and often also on the chemical shift timescale. As a consequence, the diffusion coefficients measured through the resonances of the free and bound chelates are equal to D_f and D_b, respectively. Moreover, the signal of the bound chelate is usually broad and has a low intensity making it difficult to distinguish from the noise. In such a case, competition experiments with other ligands of medium affinity and sharing the same binding site are useful [41–43]. This approach has theoretically the additional advantage of providing information about the binding site of the studied compound. In this work, we performed competition experiments with ibuprofen, which binds to Suddlow site II, as the primary site, and to other secondary sites [24], and with salicylate, which binds mainly to Suddlow site I but also with secondary sites in Suddlow site II [23].

As a third technique we used electrospray mass spectrometry to obtain mass spectra of HSA in the presence of various contrast agent concentrations. Electrospray ionization mass spectrometry (ESI-MS) is emerging as a new method to study biomolecular non-covalent interactions [19,40,44,45]. The ability of ESI-MS to study specific non-covalent complexes originates from its soft ionization, which does not induce any unwanted molecular fragmentation thus allowing weak non-covalent interactions to survive the electrospray process. Interaction between the contrast agent and HSA induces peaks corresponding to the supramolecular complex, in addition to the peaks corresponding to the free HSA. This method gives direct information not only on the affinity of the contrast agent for HSA but also on the stoichiometry of the complex [19,40].

2. Materials and methods

2.1. Chemicals

1-*p*-isothiocyanatobenzyldiethylenetriaminepentaacetic acid was obtained from Macrocyclics (Dallas, USA). Sulfaphenazole and human serum albumin (HSA, product no. A-1653, powder 96–99%) were purchased from Sigma (Bornem, Belgium). HSA was used without further purification for the relaxometric and the diffusion measurements. For mass spectrometry experiments, HSA was however desalted by five dilution-concentration steps using Microcon YM-10 from Millipore (Brussels, Belgium). The protein concentration was measured spectrophotometrically (UV $\lambda = 280$ nm) on an 8452A diode array spectrophotometer (Hewlett–Packard, Brussels, Belgium). The HSA concentration was 0.6 mM (4%) for the proton relaxation rate analysis and the diffusometry technique and 5 μM for the mass spectrometry experiments. The relaxometric measurements were performed in water, except the competition experiments which were performed in a phosphate buffer (0.2 M NaH_2PO_4 + 0.2 M Na_2HPO_4, pH 7.4). The diffusion measurements were carried out in the same phosphate buffer but in heavy water and the mass spectrometry measurements were performed in ammonium acetate, which disturbs only slightly the conformation of HSA [40].

2.2. Synthesis of gadolinium and europium complexes

The reaction scheme is shown in Fig. 2. A water solution of sulfaphenazole was added to a water solution of 1-*p*-isothiocyanatobenzyldiethylenetriaminepentaacetic acid

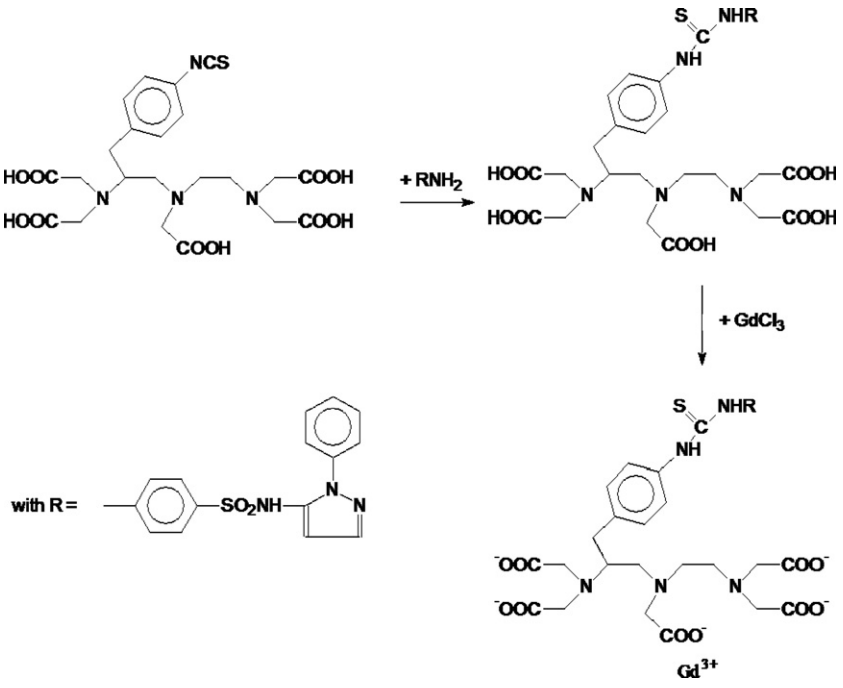

Fig. 2. Synthetic scheme of Gd–C_4-sulfaphenazol-DTPA.

(1.5 equivalents). The pH of the solution was set between 9 and 10 and the mixture was stirred during 48 h at room temperature. After reaction, the product was purified by dialysis on a Spectra/Por® Biotech Cellulose Ester (CE) membrane with a cut-off of 500 (VWR, Leuven, Belgium) and by column chromatography on silica gel 60 RP-18 (40–63 μm) (Merck, Darmstadt, Germany) with methanol/water 40/60 (v/v) as elution solvent. ^{1}H NMR (D_2O, δ (ppm), m, multiplet; s, singlet; d, doublet; pyr, pyrazole): 2.7–3.1 (m, 7H, $3 \times CH_2 + 1 \times CH$); 3.1–3.3 (m, 2H, $1 \times CH_2$); 3.6–3.7 (s, 10H, $5 \times CH_2$–COOH); 5.7 (d, 1H, $1 \times$ CH pyr.); 6.6 (d, 1H, $1 \times$ CH pyr.); 7.1–7.2 (m, 6H, $6 \times$ CH); 7.2–7.3 (m, 6H, $6 \times$ CH); 7.5–7.6 (d, 1H, $1 \times$ CH).

For the complexation, a water solution of gadolinium chloride or europium chloride (1 equivalent) was added dropwise while maintaining the pH between 6 and 8. The resulting mixture was then stirred during 24 h. The presence of free gadolinium or europium ions was detected with arsenazo III (2,7-bis(arsonophenylazo)-1,8-dihydroxynaphthalene-3,6-disulfonic acid, Aldrich, Bornem, Belgium), a complexometric indicator [46]. The free ions were removed with Chelex® 100 (Fluka, Bornem, Belgium). The complex was then dialyzed using a membrane with an MWCO of 500. Eu-complex: ES-MS $(M + 2Na)^+$: 1047, 1049 (Eu isotopes); $(M + 3Na)^+$: 1069, 1071; $(M + 4Na)^+$: 1091, 1093.

2.3. Proton relaxation rate analysis

Proton relaxation rates were measured at 0.47 T and 310 K on Minispecs PC-20 or mq-20 (Bruker, Karlsruhe, Germany) by a standard inversion-recovery sequence.

Proton nuclear magnetic relaxation dispersion (NMRD) profiles were recorded on a Stelar Relaxometer (Mede, Italy) working between 0.24 mT and 0.24 T. The additional relaxation rates at 0.47 T, 1.41 T, and 7.05 T were measured on mq-20 and mq-60 Minispec systems (Bruker), and on an AMX300 spectrometer (Bruker). The transmetallation measurements by Zn^{2+} ions were performed at 310 K and 0.47 T with a concentration of gadolinium complex and of Zn^{2+} ions of 2.5 mM, in a phosphate buffer ($[KH_2PO_4] = 0.026$ mol/L and $[Na_2HPO_4] = 0.041$ mol/L) [47].

2.4. NMR diffusion measurements

The measurements were performed on an Avance200 spectrometer equipped with a variable temperature high resolution diffusion probe (Bruker). A "PGSE" (pulsed gradient spin echo) sequence with $\Delta = 4$ ms and $\delta = 1$ ms was used. The gradient strength was calibrated with water ($D = 3 \times 10^{-9}$ m^2/s at 310 K) and the maximum gradient strength was 800 G/cm. The temperature was maintained at 310 K by circulation of water in the gradient coil (water bath HAAKE UWK 45). All the solutions were prepared in a phosphate buffer made with D_2O. The diffusion coefficients were obtained by a fit of the peak heights, manually measured, versus the field gradient using the following equation:

$$I = I_0 \exp(-\gamma^2 g^2 D \delta^2 (\Delta - \delta/3)) \quad (3)$$

A biexponential fit of the data, where one of the components was assumed to be governed by the diffusion coefficient of HSA, was necessary in order to calculate the diffusion coefficient of ibuprofen or salicylate because of a signal overlap between the ligand and the HSA. Depending on the concentration, the number of scans varied between 400 and 20,000.

2.5. Electrospray mass spectrometry measurements

Electrospray mass spectra were obtained on a Q-tof 2 (Micromass, Manchester, UK) in the positive ion mode at a capillary voltage of 1.4 kV. Samples, dissolved in ammonium acetate (100 mM), were injected with needles at a flow rate of a few nL/min [40]. Each spectrum is the sum of approximately 400 scans. The raw spectra were then baseline-corrected before deconvolution, using the program MaxEnt1™. The concentration of albumin samples injected in the mass spectrometer was 5 μM. All spectra were recorded at a cone voltage of 180 V and a source temperature of 353 K. The concentration of HSA was fixed at 5 μM in ammonium acetate and the concentration of contrast agent ranged from 5 to 50 μM.

3. Results and discussion

3.1. Water solution

The efficacy of an MRI contrast agent depends on its magnetic interaction with water protons. The classical description takes into account two contributions: the inner-sphere model described by Solomon [48] and Bloembergen [49], which refers to short distance interactions, and the outersphere contribution described by Freed [50], which accounts for the larger distance interactions. Various parameters related to the structure and the dynamics of the complex are used in these models. Some of them are quite similar for all low molecular weight complexes, like the distance of closest approach ($d = 0.36$ nm), the distance between the protons of the coordinated water molecules and the gadolinium ($r = 0.30$–0.31 nm), and the relative diffusion constant which is close to the value of pure water ($D = 3.0 \times 10^{-9}$ m^2 s^{-1} at 310 K) [51]. The structure of the complex determines other parameters, i.e., q, the number of coordinated water molecules; τ_M, the residence time of these water molecules; τ_R, the rotational correlation time of the complex; τ_{SO}, the electronic relaxation time at very low fields; and τ_V, the correlation time describing the modulation of the zero field splitting (ZFS).

Considering its structure, the number of coordinated water molecules of our complex was assumed to be equal to one. The higher boundary value of τ_M can be estimated

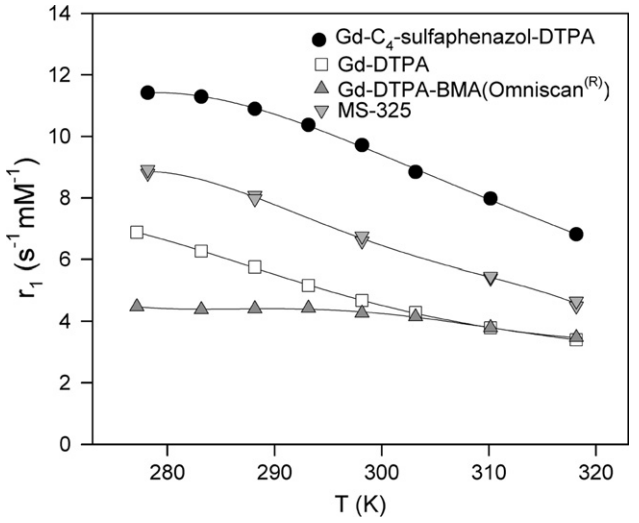

Fig. 3. Evolution of the water proton relaxivity of the Gd–C_4-sulfaphenazol-DTPA with temperature ($B_0 = 0.47$ T). The curves for Gd–DTPA, Gd–DTPA–BMA (Omniscan®), and MS-325, which where shown to have τ_M values at 310 K of 143 ns, 967–1025 ns, and 83 ns, respectively [7,18,52], are added for comparison.

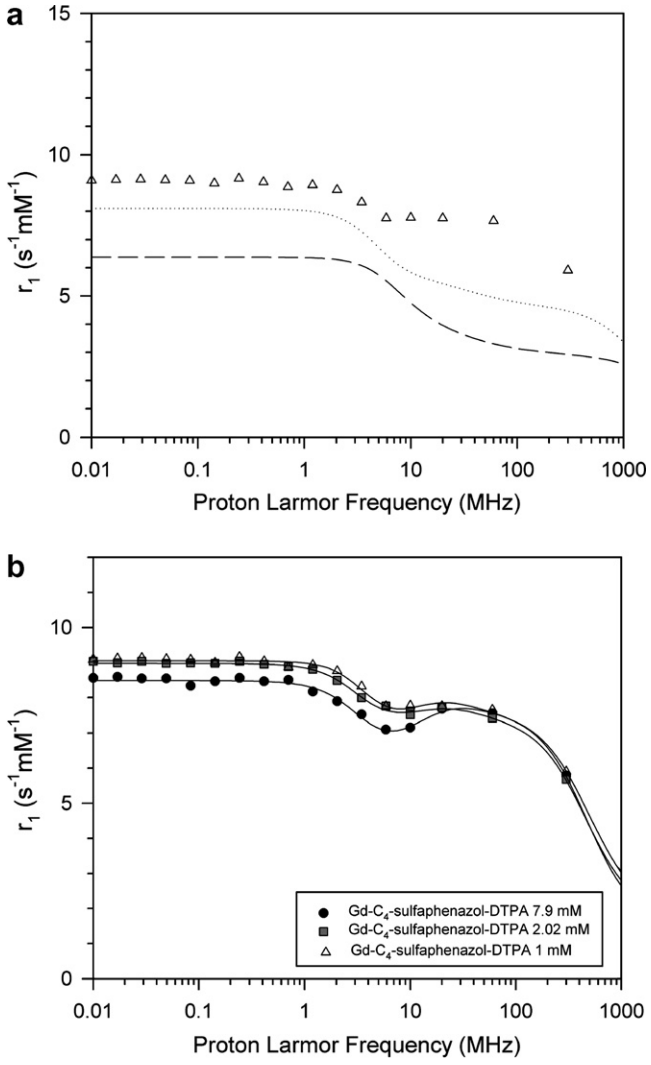

Fig. 4. NMRD profiles of Gd–C_4-sulfaphenazol-DTPA at 310 K. (a) Gd–C_4-sulfaphenazol-DTPA 1 mM. The NMRD profiles of Gd–DTPA (dashed line) and of MS-325 (dotted line) have been added for comparison. (b) NMRD profiles at three different concentrations. The lines through the data aim at guiding the eye.

from the evolution of the proton relaxivity (defined as the increase of the water relaxation rate induced by 1 mmol/L of complex) versus temperature. The continuous increase of the relaxivity between 318 K ($r_1 = 6.8\ s^{-1}\ mM^{-1}$) and 278 K ($r_1 = 11.4\ s^{-1}\ mM^{-1}$) is typical of a fast exchange between the coordinated water molecule and bulk water (Fig. 3). Indeed, the evolution of the water proton relaxivity with temperature for MS-325, which was reported to have a τ_M of 83 ns at 310 K, is similar to that of our compound [7,8]. On the contrary, the data observed for Gd–DTPA–BMA (gadodiamide, Omniscan®; Nycomed, Oslo, Norway), which has a τ_M of about 1 μs at 310 K [18,52], clearly shows a quenching of the relaxivity at low temperature. A relatively short value of τ_M at 310 K ($\tau_M^{310} \leq 100$ ns) can thus be assumed for our complex. It should be noted that such a τ_M value has very little influence on the proton relaxivities of small or medium size complexes at 310 K.

The proton NMRD profile of the complex (1 mM solution) was recorded at 310 K and compared to the NMRD profiles of the parent compound Gd–DTPA and of MS-325 (Fig. 4a). The observed relaxivity of our new complex is larger than for the parent compounds Gd–DTPA and MS-325 ($r_1 = 7.8$ versus 3.85 and $5.45\ s^{-1}\ mM^{-1}$ at 0.47 T, respectively) and unexpectedly high above 5 MHz. Based on the molecular weights and on the similarity of the chemical structures of MS-325 and our complex, similar NMRD profiles were however expected. The differences observed at high field result from an increase of the rotational correlation time and could be explained by the formation of aggregate by Π-stacking between the molecules in the solution. NMRD profiles recorded at several concentrations (1 mM, 2 mM, and 7.9 mM) (Fig. 4b) show a hump at high fields for the larger concentrations, which agrees with the possible formation of aggregates resulting from the intermolecular interaction between the aromatic parts of the molecules.

The stability of the new complex was tested by measuring the exchange between the gadolinium ion and the zinc ion, which is known as the blood ion most likely to be able to exchange with the gadolinium ion in plasma because of its similar radius. This experiment was performed in the presence of phosphate ions, which form an insoluble complex with the gadolinium ions. During the transmetallation process, the released gadolinium ions precipitate with the phosphate ions and they no longer contribute to the proton paramagnetic relaxation rate of the solution. As a result, the paramagnetic relaxation rate of the solution decreases [47]. For Gd–C_4-sulfaphenazol-DTPA, a decrease of 30% of the proton paramagnetic relaxation rate is observed after 100 h, attesting to a very good stability of the

complex. Its stability is slightly better than that of Gd–EOB–DTPA (Primovist™), markedly higher as compared to Gd–DTPA and Gd–DTPA–BMA (Omniscan®) [47] and comparable to that of MS-325 [7] (Fig. 5).

3.2. HSA solution

3.2.1. Proton relaxation rate analysis

The NMRD profile of the complex at a concentration of 1 mM in the presence of HSA 4% (Fig. 6) is characterized by a large hump at high fields (between 10 and 60 MHz), reflecting the increase of the rotational correlation time (τ_R) of the complex, which is the consequence of its non-covalent interaction with HSA. The apparent maximum relaxivity, i.e. the paramagnetic relaxation rate of the 1 mM solution in HSA 4%, is quite high and close to 25 s^{-1} mM^{-1}, a value similar to that found for MS-325 [7,8].

Quantification of the affinity of this contrast agent for HSA was attempted by proton relaxation rate analysis at 20 MHz, which is in the frequency range of the NMRD profile where the effect of the interaction is maximal (Fig. 7a). The fitting of the data using Eq. (1), fixing the lower limit of r_1^c as the largest values of the apparent relaxivity (defined as R_1^P/[Gd-complex]), provides a K_a of about 5470 ± 4540 M^{-1} with 2.2 ± 0.8 binding sites and an r_1^c of 30 ± 0.5 s^{-1} mM^{-1}. This K_a value is lower than that reported for sulfaphenazole [25–28] and suffers from the hypothesis of identical and independent binding sites and

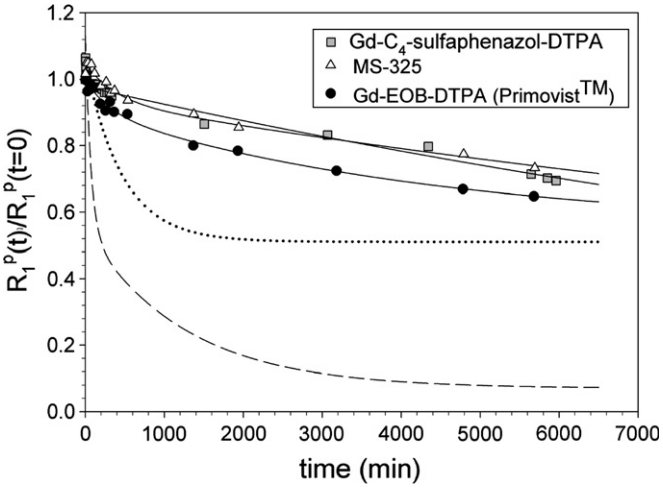

Fig. 5. Comparison of the evolution of the paramagnetic proton relaxation rate during transmetallation of Gd–EOB–DTPA (Primovist™) [47], MS-325 [7], and Gd–C_4-sulfaphenazol-DTPA by Zn(II) ions ($B_0 = 0.47$ T, $T = 310$ K, pH 7)). Data previously obtained for Gd–DTPA [47] (dotted line) and Gd–DTPA–BMA (Omniscan®) [47] (dashed line) are added for comparison.

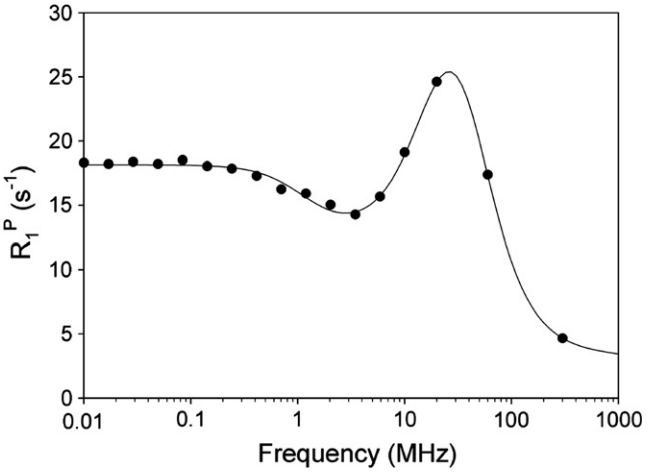

Fig. 6. NMRD profile of the complex at a concentration of 1 mM in the presence of 4% HSA at 310 K. The line through the data aims at guiding the eye.

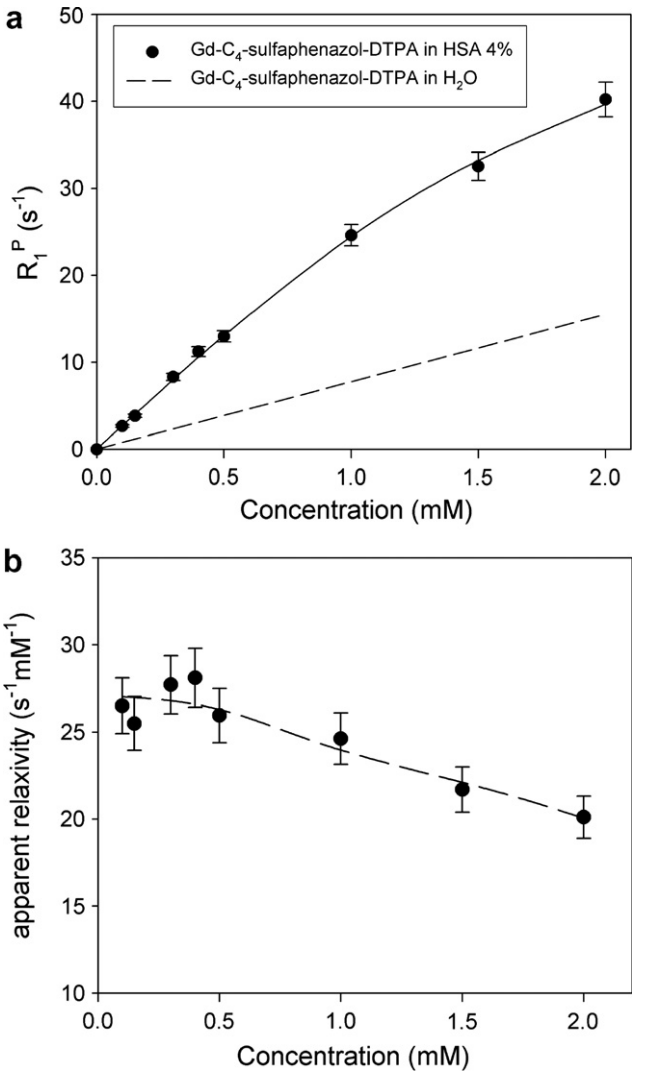

Fig. 7. (a) Evolution of the paramagnetic relaxation rate of water protons in the presence of various concentrations of the gadolinium complex and of a 0.6 mM concentration of HSA ($T = 310$ K, $B_0 = 0.47$ T). (b) Apparent relaxivities calculated from the data in Fig. 7a. The dashed line was calculated assuming one strong binding site ($K_{a1} = 7.5 \times 10^5$ M^{-1}) with a relaxivity of 27.5 s^{-1} mM^{-1} and two sites of weaker affinity ($K_{a2} = 2 \times 10^4$ M^{-1}) with a relaxivity of 20 s^{-1} mM^{-1}.

from a lack of precision because of the number of undetermined and correlated parameters in Eq. (1): indeed, K_a, n, and the relaxivity of the complex HSA–contrast agent (r_1^c) are strongly correlated.

The apparent relaxivities calculated from the data in Fig. 7a remain approximately constant at a value close to 27 s^{-1} mM^{-1} in the concentration range extending from 0.1 mM to 0.5 mM and then decrease as the contrast agent concentration increases, due to the lessening of the bound fraction of the Gd-complex (Fig. 7b). These data can be explained by the presence of a strong binding site and additional weaker binding sites. Indeed, the experimental data could be fitted using the assumption of one strong binding site ($K_{a1} = 7.5 \times 10^5$ M^{-1}) with a relaxivity of 27.5 s^{-1} mM^{-1} and two sites of weaker affinity ($K_{a2} = 2 \times 10^4$ M^{-1}) with a relaxivity of 20 s^{-1} mM^{-1} (see dashed line in Fig. 7b). The resulting 1:1 HSA/contrast agent complex has however a moderate relaxivity that could be explained by a conformation that does not allow a very fast exchange of the coordinated water with the bulk or by some displacement of the coordinated water by other coordinating groups of the binding site.

3.2.2. NMR diffusometry

NMR diffusometry was performed on the europium complex as explained above (see Section 1). During the titration experiment, i.e. the experiment in which the concentration of HSA is fixed to 4% and the concentration of the europium complex varies, no evolution of the diffusion coefficient was observed. This result differs from the simulated curve performed on the basis of a fast exchange between bound and free states and of the data obtained from Fig. 7b, which predicts a significant evolution of the diffusion coefficient (Fig. 8). This discrepancy can be explained by a slow exchange between albumin and the contrast agent on the diffusion timescale. The non-covalent interaction with albumin is indeed usually described as a two-step phenomenon: the first step is essentially driven by enthalpy and is rapid, while the second step is mostly driven by entropy and is very slow, corresponding to the hydrophobic interaction between HSA and the ligand. In most cases, the entropic factor is predominant, which makes the exchange between the bound and the free ligand very slow [23].

3.2.3. Electrospray mass spectrometry

The electrospray mass spectra (Fig. 9) clearly show two signals corresponding to a complex between HSA and the gadolinium complex, besides that of HSA alone, when the concentrations of HSA and of Gd–C_4-sulfaphenazol-DTPA are equal (5 μM). In addition, the lower intensities of the HSA peaks as compared to the HSA/Gd-complex peaks indicate a very strong affinity of the Gd-complex for HSA. With increasing Gd–C_4-sulfaphenazol-DTPA concentrations, the number of signals corresponding to a complex between HSA and the contrast agent increases to reach a value of five (i.e., simultaneous presence of

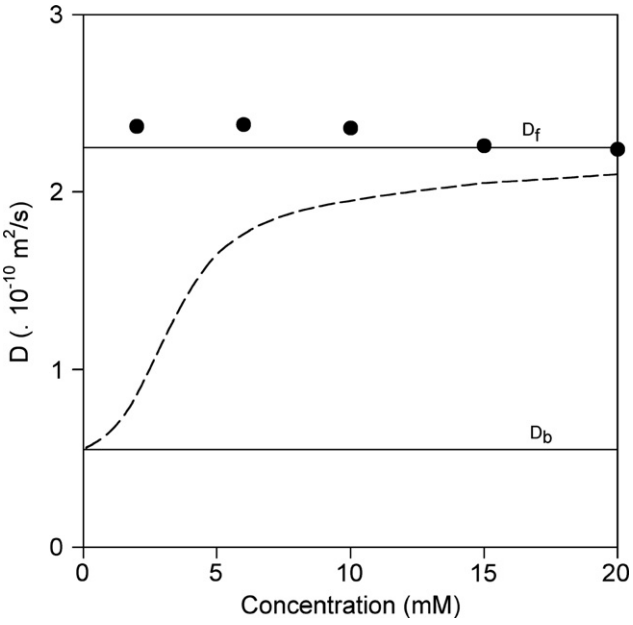

Fig. 8. Titration experiment with the NMR diffusometry technique. The closed squares represent the evolution of the diffusion coefficient of the europium complex in the presence of 4% HSA ($T = 310$ K, $B_0 = 4.7$ T)). The dashed line represents a simulation of the diffusion curve based on the results obtained by the proton relaxation rate analysis technique ($K_{a1} = 7.5 \times 10^5$ M^{-1} with $n = 1$ and $K_{a2} = 2 \times 10^4$ M^{-1} with $n = 2$) and assuming a fast exchange of the lanthanide-complex.

1:1, 1:2, 1:3, 1:4, and 1:5 HSA/Gd-complex entities) when the concentration of the contrast agent is ten times larger than the concentration of the protein. In order to obtain more quantitative information, the peak heights of the various multiply charged ions of the free HSA and of the complex were summed to evaluate the respective concentrations. This treatment was carried out using the software MaxEnt1™ which uses the maximum-entropy method to reconstruct neutral molecular mass spectra from spectra of multiply charged forms. Thanks to this treatment, the concentrations of the different species were obtained. When both concentrations of HSA and chelates are identical (5 μM), it can be calculated that 53% of the Gd-complex is bound to HSA. The corresponding K_a is 4.8×10^5 M^{-1}, a value much higher than that found for MS-325 in the same condition ($K_a = 1.9 \times 10^4$ M^{-1}) [19].

3.2.4. Competition experiments

The competition experiments with ibuprofen and salicylate were first performed with the proton relaxation rate analysis technique. Addition of 1 mM of ibuprofen or salicylate to a solution containing 1 mM of Gd-complex and 4% of HSA induced a moderate and similar decrease of the paramagnetic relaxation rate (decrease < 15%) at 20 MHz. Further increases of the salicylate concentration until 5 mM had little additional effect (maximum decrease of 16%), whereas at the same concentration (5 mM) ibuprofen induced a decrease of about 30%. Similarly,

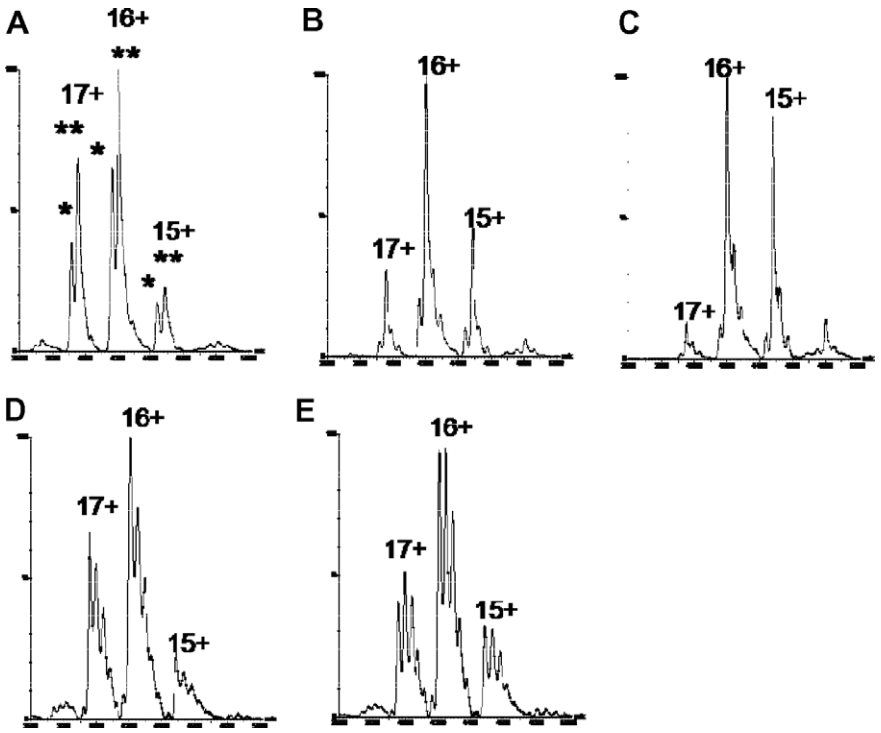

Fig. 9. Mass spectra of HSA (5 μM) in the presence of Gd–C_4-sulfaphenazol-DTPA at various concentrations: A (5 μM), B (10 μM), C (20 μM), D (30 μM), and E (50 μM). *Corresponds to the peaks of HSA and **corresponds to the peaks of the 1:1 complex HSA/Gd–chelate.

the NMRD profiles performed on solutions containing 2 mM of contrast agent, 4% of HSA, and 10 mM of competitors (Fig. 10) show a larger decrease of the paramagnetic relaxation rate when ibuprofen is present. Ibuprofen seems thus to be a more efficient competitor of the contrast agent than salicylate.

Competition was also assessed through the diffusometry technique performed on solutions containing 2 mM of europium complex, 4% of HSA, and 10 mM of ibuprofen or salicylate. In these experiments, the diffusion coefficient of the competitor, i.e. ibuprofen or salicylate, was measured. Titration experiments were first performed for each of the competitors (data not shown) and reflect only the secondary binding sites of the ligands. As described in the introduction, if the interaction with the main site is quite strong, the exchange will be too slow to observe an evolution of the diffusion coefficient. However, if the competitor and the Eu-complex interact with the same primary or secondary site(s), the competitor will be displaced from its binding sites and consequently its diffusion coefficient is expected to increase. This is the case for ibuprofen, for which the diffusion coefficient increases from 3.08×10^{-10} m^2/s in the absence of the Eu-complex to 3.94×10^{-10} m^2/s in its presence, whereas no significant evolution of the diffusion coefficient of salicylate is observed (D salicylate $= 5.34 \times 10^{-10}$ m^2/s and 5.17×10^{-10} m^2/s before and after the addition of the Eu-complex, respectively) (Fig. 11). The increase of the diffusion coefficient of ibuprofen corresponds to an increase of the concentration of free ibuprofen from about 6 mM to about 8 mM (concentrations estimated using Eq. (2) with $D_f = 4.52 \times 10^{-10}$ m^2/s and $D_b = 0.55 \times 10^{-10}$ m^2/s). From these results, it appears that the Eu-complex (2 mM) displaced ibuprofen from its binding sites ($K_{a1} = 2.73 \times 10^6$ M^{-1} ($n = 1$), $K_{a2} = 1.95 \times 10^4$ M^{-1} ($n = 6$–7)) [20]. These data confirm the strong interaction of our complex with HSA, as shown by the electrospray mass experiments and suggested by the first diffusometry results (Fig. 8) and by the evolution of the apparent relaxivity (Fig. 7b).

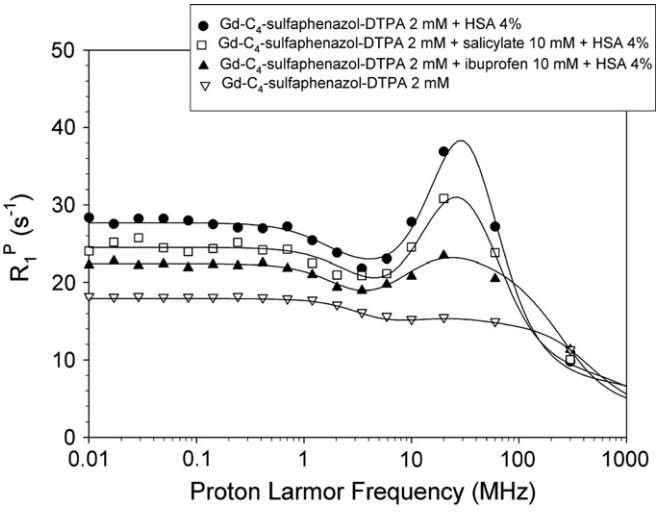

Fig. 10. NMRD profiles of 2 mM Gd–C_4-sulfaphenazol-DTPA: alone in an aqueous buffer, in the presence of 4% HSA, and in the presence of 4% HSA and of each of the competitors ($T = 310$ K). The lines through the data aim at guiding the eye.

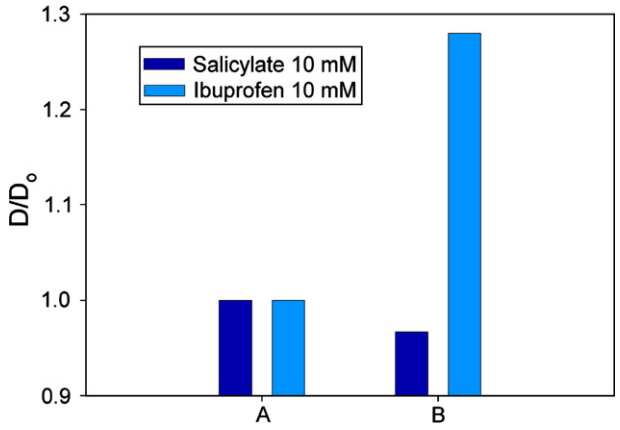

Fig. 11. Competition experiments with ibuprofen and salicylate by the NMR diffusometry technique. A corresponds to the diffusion coefficients of the competitors (D_0 ibuprofen $= 3.08 \times 10^{-10}$ m^2/s and D_0 salicylate $= 5.34 \times 10^{-10}$ m^2/s) in HSA solution, which were normalized to 1, while B corresponds to the relative diffusion coefficients of the competitors in the presence of HSA and of 2 mM Eu-C$_4$-sulfaphenazol-DTPA (D/D_0) (D ibuprofen $= 3.94 \times 10^{-10}$ m^2/s and D salicylate $= 5.17 \times 10^{-10}$ m^2/s).

During the competition experiments performed using the proton relaxation rate analysis technique, high concentrations of salicylate could have partly displaced the Gd-complex from the secondary sites, inducing a small decrease of R_1^P, whereas ibuprofen possibly decreased the binding of the chelate not only from its secondary sites but also from its primary site. During the competition experiments carried out using the diffusometry technique (10 mM of salicylate and 2 mM of the Eu-complex), the lanthanide-complex concentration is probably too low to significantly displace salicylate from its binding sites (the reported association constants of salicylate are: $K_{a1} = 2.2 \times 10^5$ ($n = 1$), $K_{a2} = 1.6 \times 10^3$ ($n = 5$)) [20]. On the contrary, the affinity of the chelate seems to be sufficiently strong to compete for ibuprofen since 2 mM of Eu-complex displace about 2 mM of ibuprofen from its binding sites.

4. Conclusions

Gd–C$_4$-sulfaphenazol-DTPA, a new gadolinium complex is characterized by a high water relaxivity, a fast water exchange, and a very good stability versus zinc transmetallation. The study of its interaction with HSA was performed using three techniques: proton relaxation rate analysis, electrospray mass spectrometry, and NMR diffusometry. In addition to the rough estimation of the association constant and of the number of binding sites, the simple proton relaxation rate analysis method gives direct information about the efficacy of the contrast agent in the presence of HSA on a wide range of magnetic fields. Nevertheless, it has to be pointed out that this technique suffers from several drawbacks due to (i) the strong correlation between the fitted association constant, the number of sites, and the relaxivity in the bound state, (ii) the assumption of equivalent and identical binding sites characterized by an hypothetic similar relaxivity, and (iii) the possible very low relaxivity of the bound complex if the coordinated water molecule is either exchanging very slowly with the bulk or replaced by a coordinating group present in the binding site. Some improvement can however be provided by the analysis of the apparent relaxivity which gives information on the possible coexistence of high affinity sites and additional sites with weaker affinity. The electrospray mass spectrometry gives direct information on the affinity of the chelate for the protein and on the stoichiometry of the complex, which is remarkable since the other methods give this information indirectly from a theoretical fitting of the data. The titration experiment with the diffusometry technique needs the use of an Eu analogue and do not provide information about the affinity of the contrast agent for HSA, however, it gives some indirect information about the exchange kinetics of the complex: the diffusion delay (Δ) of 4 ms does not allow the exchange of the lanthanide-complex between the bound and the free forms. We can thus conclude that the complex's lifetime is of the order or higher than 4 ms, in agreement with published data [53–55]. In addition, the diffusometry technique competition experiments show a competition between ibuprofen and the Eu-complex.

The results obtained by the three techniques are compatible with a strong interaction of the Gd-complex with HSA. This new complex, therefore, appears as a potential MRI contrast agent. Further *in vivo* experiments (pharmacokinetics, biodistribution, MRI) will be undertaken in a near future.

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References

[1] R.N. Muller, in: D.M. Grant, R.K. Harris (Eds.), Encyclopedia of Nuclear Magnetic Resonance, Wiley and Sons, New York, 1996, pp. 1438–1444.
[2] R.B. Lauffer, Chem. Rev. 87 (1987) 901–927.
[3] E. Toth, L. Helm, A.E. Merbach, in: A.E. Merbach, E. Toth (Eds.), The Chemistry of Contrast Agents in Medical Magnetic Resonance Imaging, Wiley, New York, 2001, pp. 46–119.
[4] M. Rohrer, H. Bauer, J. Mintorovitch, M. Requardt, H.-J. Weinmann, Invest. Radiol. 40 (11) (2005) 715–724.
[5] S. Aime, M. Botta, E. Terreno, Adv. Inorg. Chem. 57 (2005) 173–237.
[6] S. Aime, S. Geninatti Crich, E. Gianolio, G.B. Giovenzana, L. Tei, E. Terreno, Coord. Chem. Rev. 250 (2006) 1562–1579.
[7] R.N. Muller, B. Radüchel, S. Laurent, J. Platzek, C. Piérart, P. Mareski, L. Vander Elst, Eur. J. Inorg. Chem. (1999) 1949–1955.

[8] S.G. Zech, H.B. Eldredge, M.P. Lowe, P. Caravan, Inorg. Chem. 46 (9) (2007) 3576–3584.
[9] P. Caravan, N.J. Cloutier, M.T. Greenfield, S.A. McDermid, S.U. Dunham, J.W.M. Bulte, J.C. Amedio Jr., R.J. Looby, R.M. Supkowski, W. DeW. Horrocks Jr., T.J. McMurry, R.B. Lauffer, J. Am. Chem. Soc. 124 (12) (2002) 3152–3162.
[10] R.A. Wallace, J.P. Haar Jr., D.B. Miller, S.R. Woulfe, J.A. Polta, K.P. Galen, M.R. Hynes, K. Adzamli, Magn. Reson. Med. 40 (1998) 733–739.
[11] K. Adzamli, L. Vander Elst, S. Laurent, R.N. Muller, Magn. Reson. Mater. Phys. Biol. Med. 12 (2001) 92–95.
[12] K. Adzamli, M. Spiller, S.H. Koenig, Acad. Radiol. 9 (suppl. 1) (2002) S11–S16.
[13] L. Vander Elst, F. Chapelle, S. Laurent, R.N. Muller, J. Biol. Inorg. Chem. 6 (2001) 196–200.
[14] L. Vander Elst, F. Maton, S. Laurent, F. Seghi, F. Chapelle, R.N. Muller, Magn. Reson. Med. 38 (1997) 604–614.
[15] S. Aime, M. Botta, M. Fasano, S.G. Crich, E. Terreno, J. Biol. Inorg. Chem. 1 (1996) 312–319.
[16] M.K. Thompson, D.M.J. Doble, L.S. Tso, S. Barra, M. Botta, S. Aime, K.N. Raymond, Inorg. Chem. 43 (2004) 8577–8586.
[17] F.M. Cavagna, F. Maggioni, P.M. Castelli, M. Dapra, L.G. Imperatori, V. Lorusso, B.G. Jenkins, Invest. Radiol. 32 (1997) 780–796.
[18] S. Laurent, L. Vander Elst, R.N. Muller, Contrast Med. Mol. Imag. 1 (2006) 128–137.
[19] V. Henrotte, L. Vander Elst, S. Laurent, R.N. Muller, J. Biol. Inorg. Chem. 12 (2007) 929–937.
[20] U. Kragh Hansen, Pharmacol. Rev. 33 (1981) 17–53.
[21] U. Kragh Hansen, Dan. Med. Bull. (1990) 37–57.
[22] D.C. Carter, J.X. Ho, Adv. Protein Chem. 45 (1994) 153–203.
[23] T. Peters Jr., All About Albumin: Biochemistry, Genetics, and Medical Applications, Academic Press, USA, 1996.
[24] J. Ghuman, P.A. Zunszain, I. Petitpas, A.A. Bhattacharya, M. Otagiri, S. Curry, J. Mol. Biol. 353 (2005) 38–52.
[25] M. Otagiri, H. Nakamura, Y. Imamura, U. Matsumoto, J. Fleitman, J.H. Perrin, Chem. Pharm. Bull. 37 (5) (1989) 1401–1403.
[26] C.F. Chignell, E.S. Vesell, D.K. Starkweather, C.M. Berlin, Clin. Pharmacol. Ther. 12 (6) (1971) 897–901.
[27] T. Fujita, J. Med. Chem. 15 (1972) 1049–1056.
[28] K.F. Brown, M.J. Crooks, Biochem. Pharmacol. 25 (1976) 1175–1178.
[29] G. Burkhard, Concepts Magn. Reson. 10 (5) (1998) 299–321.
[30] W.S. Price, Concepts Magn. Reson. 9 (5) (1997) 299–336.
[31] W.S. Price, Concepts Magn. Reson. 10 (4) (1998) 197–237.
[32] L.H. Lucas, C.K. Larive, Concepts Magn. Reson. A 20A (1) (2004) 24–41.
[33] R.S. Luo, M.L. Liu, X.A. Mao, Spectrochim. Acta A 55 (1999) 1897–1901.
[34] Z. Ji, H. Yuan, M. Liu, J. Hu, J. Pharm. Biomed. Anal. 30 (2002) 151–159.
[35] T.S. Derrick, E.F. McCord, C.K. Larive, J. Magn. Reson. 155 (2002) 217–225.
[36] Y. Ma, M. Liu, X.A. Mao, J.K. Nicholson, J.C. Lindon, Magn. Reson. Chem. 37 (1999) 269–273.
[37] R.S. Luo, M.L. Liu, X.A. Mao, Appl. Spectrosc. 53 (7) (1999) 776–779.
[38] J.C. Lindon, M. Liu, J.K. Nicholson, Rev. Anal. Chem. 18 (1–2) (1999) 23–66.
[39] M. Liu, J.K. Nicholson, J.C. Lindon, Anal. Commun. 34 (1997) 225–228.
[40] V. Henrotte, S. Laurent, V. Gabelica, L. Vander Elst, E. Depauw, R.N. Muller, Rapid Commun. Mass Spectrom. 18 (17) (2004) 1919–1924.
[41] L.H. Lucas, K.E. Price, C.K. Larive, J. Am. Chem. Soc. 126 (2004) 14258–14266.
[42] C. Dalvit, M. Flocco, S. Knapp, M. Mostardini, R. Perego, B.J. Stockman, M. Veronesi, M. Varasi, J. Am. Chem. Soc. 124 (2002) 7702–7709.
[43] C. Dalvit, M. Flocco, B.J. Stockman, M. Veronesi, Comb. Chem. High Throughput Scr. 5 (2002) 645–650.
[44] J.A. Loo, Int. J. Mass Spectrom. 200 (2000) 175–186.
[45] T.D. Veenstra, Biophys. Chem. 79 (1999) 63–79.
[46] A. Barge, G. Cravotto, E. Gianolio, F. Fedeli, Contrast Med. Mol. Imag. 1 (2006) 184–188.
[47] S. Laurent, L. Vander Elst, F. Copoix, R.N. Muller, Invest. Radiol. 36 (2001) 115–122.
[48] I. Solomon, Phys. Rev. 99 (1955) 559–565.
[49] N. Bloembergen, J. Chem. Phys. 27 (1957) 572–573.
[50] J.H. Freed, J. Chem. Phys. 68 (1978) 4034–4037.
[51] L. Vander Elst, A. Sessoye, S. Laurent, R.N. Muller, Helv. Chim. Acta 88 (3) (2005) 574–587.
[52] D.H. Powell, O.M. Ni Dhubhghaill, D. Pubanz, L. Helm, Y.S. Lebedev, W. Schlaepfer, A.E. Merbach, J. Am. Chem. Soc. 118 (1996) 9333–9346.
[53] V. Maes, Y. Engelborghs, J. Hoebeke, Y. Maras, A. Vercruysse, Mol. Pharmacol. 21 (1) (1982) 100–107.
[54] Y. Keita, W. Wörner, G. Veile, B.G. Woodcock, U. Fuhr, Arzneim.-Forsch. 46 (2) (1996) 164–168.
[55] B. Meyer, T. Peters, Angew. Chem. Int. Ed. 42 (8) (2003) 864–890.

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