Is DTPA a good competing chelating agent for Th(IV) in human serum and suitable in targeted alpha therapy?

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IS DTPA A GOOD COMPETING CHELATING AGENT FOR Th(IV) IN HUMAN SERUM AND SUITABLE IN TARGETED ALPHA THERAPY ?

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ABSTRACT

The interaction between thorium and human serum components was studied using difference ultraviolet spectroscopy (DUS), ultrafiltration and high–pressure–anion exchange chromatography (HPAEC) with external inductively conducted plasma mass spectrometry (ICP–MS) analysis. Experimental data are compared with modelling results based on the law of mass action. Human serum transferrin (HSTf) interacts strongly with Th(IV), forming a ternary complex including two synergistic carbonate anions. This complex governs Th(IV) speciation under blood serum conditions. Considering the generally used Langmuir-type model, values of $10^{33.5}$ and $10^{32.5}$ were obtained for strong and weak sites, respectively. We showed that trace amounts of diethylene triamine pentaacetic acid (DTPA) cannot complex Th(IV) in the blood serum at equilibrium. Unexpectedly this effect is not related to the

competition with HSTf but is due to the strong competition with major divalent metal ions for DTPA. However, Th-DTPA complex was shown to be stable for a few hours when it is formed before addition in the biological medium; this is related to the high kinetic stability of the complex. This makes DTPA a potential chelating agent for synthesis of ^{226}Th-labeled biomolecules for application in targeted alpha therapy.

KEYWORDS thorium; targeted alpha therapy, speciation, transferrin, DTPA

1. Introduction

The quantitative description of the interactions of thorium (IV) with blood serum components is of high relevance for the rational design of molecules suitable for *in vivo* chelation of thorium in medical applications [1, 2] or for decorporation in case of accidental ingestion [3-5]. In the first case, the chelating agent is used to couple the radionuclide to tumor selective carrier molecules to target and destroy cancer cells [6]. Here the Th(IV)-chelate complex is formed *in vitro* before application to the patient. The alpha emitters ^{226}Th ($t_{1/2}$=31 min) and ^{227}Th ($t_{1/2}$=18,7 days) are promising radionuclides for application in targeted alpha therapy (TAT) [1, 2, 7]. In the second case, the ligand should target the unwanted metal ion *in vivo* and form soluble complexes which can be excreted via the kidneys.

Thorium distribution in rats serum was studied by Peter and Lehmann in 1981 [8]. By the means of chromatographic studies and competition experiments with iron, the authors reported that the binding site of Th(IV) was the iron-binding site of transferrin (HSTf). Similar behaviour was reported for different actinides (IV) including Pu(IV) from various *in vivo* and *in vitro* studies [3, 9-11]. The interaction between Th(IV) and HSTf was further studied by Harris et al. [12] by using difference ultraviolet spectroscopy (DUS). As in the

iron-transferrin complex, two Th(IV) ions are bound to HSTf protein at the physiological pH. Two and one tyrosine groups were proposed to coordinate to thorium in strong C–terminal and weak N–terminal sites, respectively. In conclusion, HSTf appears as the main component governing Th(IV) speciation in blood serum [13]. An estimation of the complexation constants can be obtained by extrapolation from the emprical law presented by Sun et al. [14]. However, to the best of our knowledge, they have never been experimentally measured. Furthermore, one recent study showed no interaction between Th(IV) and HSTf [15], these results being rather conflicting with the others.

The first objective of the present paper is to complete available literature data to give a better comprehensive view of Th(IV) speciation in blood serum. The interaction of Th(IV) with HSTf has been studied by Difference ultraviolet spectroscopy (DUS) to determine complexation constants. The effect of human serum albumin (HSA) on Th(IV) speciation in the blood serum, the second important metallo-protein present in the biological medium, has been also estimated. Based on these parameters, and including literature data of thermodynamic constant of Th(IV) complexation with low molecular weigh organic and inorganic ligands, a model calculation was performed to describe Th(IV) interactions in blood serum. The reliability of the simulation is considered regarding the published *in vivo* experimental speciation results [8] and the experimental data from the speciation studies realized by High Pressure Anion Exchange Chromatography (HPAEC) with inductively conducted plasma mass spectrometry (ICP–MS) detection.

In addition, the developped model is used in combination with *in vitro* studies performed by ultrafiltration, to assess the efficiency of diethylene triamine pentaacetic acid (DTPA) to form stable complexes with Th(IV) under blood serum conditions. DTPA is the chelate recommended for the decorporation of Th(IV) [16]. It is not toxic for humans and forms a strong complex with Th(IV) [17, 18]. According to the “hard and soft (Lewis) acids and

bases" (HSAB) therory [19], it presents nitrogen and oxygen atoms suitable for the complexation of the "hard" Th(IV) cation and it is well preorganised for the coordination of spherical cations. Furthermore, it can be easily attached to a biological vector; this is a pre-requisite in TAT and may be useful in chelation therapy. The obtained results are discussed to assess the potential use of DTPA as a chelating agent for TAT with ^{226}Th.

2. Materials and methods

2.1. Reagents

Human serum transferrin (HSTf, apo–transferrin human ≥ 98%, Sigma) was purified by size–exclusion chromatography on a PD–10 column using 0.1 M NaCl / 2.10^{-3} M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) at pH 7.4 as an eluent. HSTf concentration was determined spectrophotometrically at λ = 280 nm using 93000 cm^{-1} M^{-1} as a molar extinction coefficient [20]. Human serum albumin (HSA, albumin from human serum ≥ 96%, Sigma) was used as received. Human serum was supplied by Lonza and was diluted in 0.1 M NaCl solution buffered at pH 7.4 equilibrated with 1 % CO_2 gas. Th(IV) stock solution was prepared by dilution of a standard solution of ^{232}Th ($^{232}Th(NO_3)_4(H_2O)_4$) (from Prodis) to obtain a final concentration of 2.10^{-3} M. Other chemicals were analytical grade. HSTf, HSA and carbonate stock solutions were freshly prepared before use.

2.2. Samples preparation

Solutions were prepared with ultrapure water (MilliQ, 18 MΩ·cm). All experiments were performed in solution of pH 7.4 ± 0.1 and ionic strength of 0.1 (NaCl) buffered with 2.10^{-3} M (HEPES) at room temperature (20 ± 2 °C). Samples in the absence of carbonate were prepared in a glove box under 100% nitrogen atmosphere. Samples in the carbonate system were prepared in a glove box under 99/1% nitrogen/carbon dioxide atmosphere. For the

complexation study realized as a function of carbonate concentration, the carbonate ion was added to the solution prepared under 100% nitrogen atmosphere. The samples were then kept closed during the experiment to prevent equilibration with atmospheric CO_2. For the complexation study realized as a function of phosphate or nitrilotriacetic acid (NTA) concentration, the samples were prepared under normal atmosphere. Inorganic carbon concentration was determined at equilibrium by total organic carbon analysis (TOC).

2.3. Analytical tools

pH measurements were performed using a PHM220 from MeterLab. Inorganic carbon analyses were done on a TOC–V_{CSH} apparatus supplied by Shimadzu. Thorium analyses were performed by ICP–MS using a XSeries 2 provided by Thermo Fisher (sensibility limit of 0.1 ppb). UV–spectra were recorded on a UV–visible UV–2401 PC spectrophotometer from Shimadzu. The centrifugation experiments were performed using a centrifuge RC6 from Thermo Fisher at 27000 g. The HPAEC device was a Dionex UltiMate3000 system consisting of a DGP–3600 MB pump, an AS3000 auto–sampler, a TCC–3200B column oven and a diode array DAD–3000 detector. The stationary phase was a Dionex AS20 anionic exchange column (2 mm diameter x 25 cm length), with an AG20 guard column (2 mm diameter x 5 cm length). The AS20 consists of a hydrophilic polymer grafted with quaternary alkanol ammonium ions. The total capacity represented by both columns is 79 microequivalents.

2.4. Difference ultraviolet spectroscopy (DUS)

DUS is the most widely used technique to evaluate metal binding to HSTf [21]. Metal complexation was estimated from the absorbance measurements using the difference spectra of the metal–protein complexes *vs.* unmetallated apo–protein. Experimental conditions are reported in Table 1. The peak area in the range 220–270 nm were monitored to describe and

to quantify Th(IV)–HSTf interaction. Equilibrium is established when the absorbance does not change during an interval of 2 hours. The error associated to the absorbance was estimated as 0.03 based on repetitive experiments. The main advantage of the method is that the absorbance signal of HSTf is not influenced by the signals of other ligands which might interact with Th(IV) (*e.g.* carbonate). The interactions of HSA, DTPA and citrate with Th(IV) were studied using competitive method with HSTf [22].

2.5. Ultrafiltration

This technique was used for Th / HSTf (1% CO_2) and Th / serum (1% CO_2) systems to differentiate the fraction of thorium bound to low– and high–molecular weight compounds. Experiments were performed in dialysis bags (Microcon Millipore, 3 kDa) and the separation was done by centrifugation at 27000 g. Thorium concentration in the filtrates was determined by ICP–MS analysis. A blank was systematically done in parallel to assess the amount of Th(IV) adsorbed on the membrane. It was assumed that the separation time (typically 30 minutes) was not sufficient to perturb equilibrium conditions and that the concentration of Th(IV) in the filtrate represented the concentration of Th(IV) associated to the low-molecular weight compounds (e.g. OH^-, CO_3^{2-}).

2.6. HPAEC analysis

The eluent at pH 7.4 was a solution of 0.1 M NaCl buffered with 2.10^{-3} M phosphate buffered saline (PBS, that as 10–fold concentrate, contains 0.1 M of sodium phosphate (Na_3PO_4) and 9% of NaCl) equilibrated with 1 % CO_2 gas controlled by bubbling. The choice of 1 % CO_2 will be explained latter. Samples and eluent were prepared in a glove box under 99/1% nitrogen/carbon dioxide atmosphere. The anion exchanger was pre–equilibrated with the eluent before starting the experiments. Samples were injected into the column through a 50

µL sample loop injector, with a flow rate of 350 µL.min^{-1}. Proteins and serum were detected spectrophotometrically at 280 nm. Th(IV) concentration was determined from collected fractions by ICP–MS analysis.

2.7. Modelling

Computer simulations were performed with the PHREEQC program [23]. Stability constants for metal ion complexes in solution are reported in Table 2 and were taken from NEA–OECD compilation [24] (complexation of Th(IV) with inorganic ligands), IUPAC [17], NIST database [18] (Th(IV) / DTPA) and from [25] for citrate and [26] for NTA. The pKa values of sulphates and phosphates ions given by PHREEQC database were taken into account. The constants given in the NIST database at an ionic strength (I) of 0.1 M were extrapolated at zero ionic strength using the truncated Davies equation [27].

The complexation of Th(IV) with HSTf was described with the model proposed by Montavon et al. [28]. The interaction between Th(IV) and HSTf was described by using two binding sites at the protein surface ($HSTF{\equiv}_1S$ and $HSTF{\equiv}_2S$). Any metal ion considered [21], the stability constants for interaction with the binding sites differ by approximately one unit of their log K value, the difference being probably related to outer–sphere effects [22]. This ratio of 10 for K was set as a fixed parameter in the fitting of our experimental data. Consequently, the formation of the complex has been described as:

$$HSTF \equiv_{1,2} S^{-2} + Th^{4+} + n(CO_3^{2-}) \underset{\leftarrow}{\rightarrow} HSTF \equiv_{1,2} STh(CO_3)_n^{-2n+2} \qquad (1)$$

Where $HSTF{\equiv}_1S$ and $HSTF{\equiv}_2S$ represent the strong and weak binding sites, respectively, and the charge of -2 relates to the two deprotonated tyrosine groups.

Alternatively modelling was also performed using the Langmuir-type model generally used in the literature. The model takes into account the metal ion speciation and the presence of two binding sites at the protein surface:

$$Th^{4+} + HSTF \equiv_{1,2} S \underset{\leftarrow}{\overset{\rightarrow}{}} Th - HSTF \equiv_{1,2} S^{4+} \quad (2)$$

The constants log K_1=19.3 and log K_2=18.2 have been extrapolated from the empirical expression given in [29] and derived from the work presented in [14]. A similar Langmuir–type model was used to described Th(IV)/HSA interaction with the consideration of one binding site.

In conclusion, for the two studied systems Th(IV)/HSTf and Th(IV)/HSA, there is only one unknown parameter (complexation constant) to be determined. The fitting of experimental data was done "by hand".

3. Results and discussion

3.1. Binding of Th(IV) to protein

3.1.1. Binding of Th(IV) to HSTf

Complexation of Th(IV) with HSTf did not occur under physiological–type conditions (pH=7.4, 0.1 M NaCl) when precaution was taken for avoiding equilibrium with atmospheric CO_2. HSTf was not able to compete with OH^- in the main $Th(OH)_4$ and $(Th)_4(OH)_{12}^{+4}$ forms. To complex Th(IV), a synergistic anion was therefore considered in the present work, starting with the NTA, the one used by Harris et al. and Jeanson et al in previous studies [12, 15]. The second one corresponds to carbonate, CO_3^{2-} being the synergistic anion allowing the complexation with iron and other metal ions [21]. Finally, the third one is HPO_4^{2-}, phosphate ions being present in the blood serum and being known to be a strong complexing agent for Th(IV) in environmental samples [30].

Complexation as a function of NTA concentration has been first investigated. The complexation kinetic was very slow; more than 4 days were necessary to reach equilibrium between Th(IV) and HSTf. The experimental data are presented in Figure 1. The complexation with HSTf starts above 10^{-6} M to reach a maximum of complexation at about

10^{-4} M, corresponding to an absorbance at 242 nm of 0.28. The start of this increase coincides with the significant formation of $Th(L)_2^{2-}$ species (L=deprotonated NTA) in the absence of HSTf (figure 1A). Our data are coherent with the one reported by Harris et al. (figure 1B). Above 10^{-4} M, the decrease in absorbance indicates dissociation of the Th–HSTf complex arising from the increasing competition between free NTA and HSTf for Th(IV) as NTA concentration increases.

Complexation as a function of carbonates concentration has been investigated in a second step. Kinetic of complexation was more rapid than in the presence of NTA (30 hours *vs* 4 days). The experimental data are presented in Figure 2B. In the carbonate concentration range from 0 to 10^{-4} M, no complexation between Th(IV) and HSTf has been observed. The complexation with HSTf starts for a carbonate concentration above 10^{-4} M, until a plateau is reached corresponding to an absorbance of 0.47. It is worth saying that the complexation coincides with the significant formation of the ternary complex $Th(OH)_2(CO_3)_2^{2-}$ in the absence of HSTf (figure 2A).

Complexation as a function of phosphate concentration has also been studied. In the concentration range from 0 to 10^{-2} M, no complexation was observed between Th(IV) and HSTf even after 2 weeks of equilibration (data not shown). Unlike NTA and carbonate, phosphate does not act as synergistic anion.

A quantitative analysis of experimental data obtained with NTA and carbonates was done as follows: the shape of the curves was reproduced by varying the values of the complexation constants between Th(IV) and HSTf and the conversion of absorbance in concentration was done considering a proportionality according to the Lambert–Beer–law (Figures 1C and 2C). In the case of NTA system, we considered the possible formation of ThL_2^{2-}, the main species existing under our conditions. A curve similar to that experimentally observed in the presence of carbonate is predicted with the occurrence of a plateau for the highest NTA concentrations

studied. The decrease of complexation observed above NTA concentration of 10^{-4} M can be quantitatively explained considering the complexation of HSTf with one Th(IV) and one ligand L^{3-}. A good agreement between the experiment and the modelling was obtained with $K_1 = 10^{31}$ and $K_2 = 10^{30}$, provided that the maximum of complexation / absorbance observed corresponds to 40 % of Th(IV) complexed to the protein (figure 1C).

For the carbonate series, we first considered the formation of a ternary complex involving one carbonate ion, as it is the case for Fe(III) or U(VI) [21, 28]. The increase of complexation can be relatively well reproduced till a carbonate concentration of 10^{-3} M with stability constants of log K_1 = 27 and log K_2 = 26 for strong [31] and weak sites, respectively. At higher carbonate concentrations, the fraction of HSTf bound to Th(IV) is expected to decrease, due to increasing competition of carbonate with HSTf for Th(IV) complexation leading to the complex $Th(OH)_2(CO_3)_2^{2-}$ (dotted line in Figure 2C). A much better agreement was obtained with two carbonate ions acting as synergistic anions: both the increase of the curve and the plateau were well described by simulation with log K_1 = 33.5 and log K_2 = 32.5. This complexation might result from the complexation of the existing species $Th(OH)_2(CO_3)_2^{2-}$ after release of two hydroxide groups. It is important to notice, that the agreement between the experiment and the theory is good provided that we consider 24% of Th(IV) not bound to the protein on the plateau (figure 2C). This was confirmed experimentally by ultrafiltration experiments; 17 ± 2 % of Th(IV) passed through the membrane and was therefore not bound to the protein.

The analysis of the data shows that the nature of the ternary complexes and the associated stability constants rely on the quantitative description of Th(IV) interaction with low-molecular weight compounds. We can finally calculate for the complexes 2:1 Th(IV):HSTf apparent absorptivities normalized with respect to HSTf concentration ($\Delta\varepsilon_{obs}$) of = 60±7 and 65±8 × 10^3 M^{-1} cm^{-1} for carbonate and NTA systems, respectively. The value obtained for the

carbonate system was used to simulate the titration curve of HSTf with Th (Figure 2D). The determination of similar values, within experimental errors, is in agreement with the fact that the absorption band is linked to the interaction of the metal ion to the phenolic groups of the tyrosine residues which is expected similar in the two studied systems. The difference in type and number of synergistic anions coordinated in the first coordination sphere do not contribute to the extinction coefficient.

Interaction of Th(IV) with HSTf, compared to the interaction U(VI)-HSTf or Fe(III)–HSTf, seems to be different. First, two carbonates are necessary to bind to Th(IV), whereas only one synergistic ion is necessary for most the metal ions, like Fe(III) or U(VI). Secondly, kinetics of complexation at HSTf surface are slow for Th(IV), whereas they are rapid for Fe(III) or U(VI) (only 20 min for U(VI) binding *vs* 30 h for Th(IV)) [28, 31]. It is worth saying that the absence of complexation between Th(IV) and transferrin observed by Jeanson et al [15] is certainly due to a kinetic problem; Th and HSTf were contacted only for a few hours. Several parameters may explain these differences between Th(IV) and Fe(III) binding. The ionic radius, for example, being much higher for Th (r = 105 pm) than for Fe (r = 65 pm) [32], the penetration of Th(IV) into the lobe of the protein may be more difficult resulting into slower kinetics. Moreover, the coordination number is 8 for Th(IV), whereas it is of 6 for Fe. In both the N– and C–terminal binding sites of HSTf, the metal ion is coordinated by four groups: two tyrosines, one histidine, and one aspartic acid providing four coordination sites for the metal. Th(IV) with a coordination number of 8, needs to complete its coordination sphere with four other coordinating groups, e.g. by two synergistic carbonates, in a bidendate mode of coordination. Fe, to complete its coordination sphere, needs only one carbonate. The occurrence of only one synergistic anion coordinated in the presence of NTA may result from steric effects, NTA being larger than carbonate.

The experiments reported in the following part have been realized at a carbonate concentration of 5.10^{-3} M (1 % CO_2), where maximal complexation between Th(IV) and HSTf occurs.

3.1.2. Binding of Th(IV) to HSA

The interaction between Th(IV) and HSA has been studied using the DUS competition method in the presence of HSTf. This method is applicable for [HSA]<8.10^{-5} M, corresponding to about one eigth of the HSA concentration found in human serum. Above HSA concentrations of 8.10^{-5} M, the DUS competition method was no more applicable because the signal to noise ratio became too low. Experimental data are shown in Figure 3. In this range of HSA concentration, no significant complexation between Th(IV) and HSA was observed; although the absorbance of the Th(IV)-HSTf complex shows a decreasing trend with increase of [HSA], the decrease was not significant within experimental errors. The data show that interaction of Th(IV) with HSTf is stronger than with HSA: in the presence of eight times more HSA than of HSTf, Th(IV) remains bound to HSTf.

3.2. Th(IV) speciation in human serum

As mentioned in the introduction, the behaviour of Th in the presence of proteins and in serum has been studied by HPAEC with external ICP–MS analysis. Usual hyphenated techniques of speciation of trace elements in biological environment were reviewed by Lobinski et al. [33]. The data with good sensitivity were obtained, more often, by using the size–exclusion or reversed phase HPLC chromatography allowing species separation. Having also interest to identify free Th or complexed Th chemical forms, we have been working on anion–exchange chromatography with the detection done by ICP–MS general method.

On the basis of the results presented in the previous section, we expect that Th(IV) is mainly interacting with HSTf in human serum. An attempt to confirm this result is presented in this section both experimentally by HPAEC and theoretically.

3.2.1. HPAEC–ICP–MS results

Under non–perturbing conditions (the eluent corresponds to a physiological-type medium; pH=7.4, 0.1 M NaCl), the method allows the separation of HSA and HSTf proteins: HSTf is not retained (elution time = 2.5 min) while HSA is eluted as a broad peak at 10.5 min. The method has been tested for Th(IV) speciation in blood serum using physiological-type media.

Figure 4 shows UV– and ICP–MS–chromatograms obtained with the different samples described in Table 1. Experiments were first realised in physiological–type media where the $Th(OH)_2(CO_3)_2^{2-}$ complex is the major Th(IV) species in solution. Only 0.5 % of Th(IV) could be eluted and no significant peak has been attributed to $Th(OH)_2(CO_3)_2^{2-}$ or detected in the time scale studied (1h30). A cleaning method with acidic solution (1 M HCl) was used to eliminate not restored Th(IV) after each experiment. The origin of this strong interaction is not known, but it will modify the equilibrium conditions controlling Th(IV) speciation in the injected solutions, as shown below.

For the injection of the solution of Th(IV) pre-equilibrated with HSTf, the Th(IV) peak was detected at the position corresponding to the one of HSTf (Figure 4A,D). This corresponds to the direct signature of the complex formed and identified by DUS. However, only 6% of Th(IV) could be recovered (96% of this fraction being present with HSTf), while 80% of Th(IV) was initially bound to HSTf in the injected solution. A similar result was obtained in the presence of HSA, Th(IV) being eluted at the retention time of HSA between 10 and 15 min (Figure 4A,D). 0.5% of the total concentration was recovered; 80% of this Th(IV) was found to be bound to HSA. Both Th(IV)–HSTf and Th(IV)–HSA complexes can therefore be

identified by HPAEC. The interaction strength between Th(IV) and the proteins is strong enough for avoiding a complete sorption of $Th(OH)_2(CO_3)_2^{2-}$ by equilibrium displacement on the resin. The percentage of eluted Th(IV) being less in the presence of HSA than in the presence of HSTf indicates that the stability constant is higher for HSTf than for HSA, in agreement with the results of the previous section.

Th(IV) was next mixed with both proteins (HSTf and HSA) (Figure 4B,E). HSTf and HSA concentrations were fixed to mimic proteins concentrations in human serum (see Table 1). As in previous experiments, only a few percent of Th(IV) were eluted (10.5 %). 85% of Th(IV) content was found to be bound to HSTf, the rest was in interaction with HSA (15%). This distribution can be quantitatively calculated using the complexation constants describing Th(IV)/HSTf interaction and considering a complexation constant of $10^{17.5}$ for describing Th(IV)/HSA interaction. This estimated value was next used for the treatment of DUS experimental data (Figure 3A). As shown in Fig 3A, a slight decrease of the percentage of the Th(IV)–HSTf is predicted as the HSA concentration increases, although this trend could not be experimentally proven because of too high uncertainties associated to the absorbance measurements.

Th(IV) speciation was next studied in human blood serum corresponding to the "real" biological system. After equilibration in the biological media for 24 hours, Th(IV) was eluted at the retention time between 3 and 5 min (9% eluted). Th(IV) appears to be well in interaction with HSTf, nevertheless no interaction with HSA was determined contrary to the observation in the synthetic system of Th(IV)/HSTf/HSA. This result is in agreement with Peter and Lehmann's work reported that the Th was bound to HSTf in the serum of rat [8].

3.2.2. Modelling

Both data obtained *in vivo* as well as the results of our study identify HSTf as the key

component governing Th(IV) speciation in blood serum. Similar to the study published on speciation of uranium in human blood serum [34], we wanted to verify these experimental results for trace concentrations of Th(IV) (10^{-9} M) in a predictive way by using the law of mass action principle. Due to the complexity of the human serum, the following simplifications were considered on the basis that the interaction strength of given serum components with Th(IV) will depend on their concentrations as well as on their equilibrium complexation constants:

(i) all relevant inorganic ligands were taken into account (hydroxide, carbonate, phosphate, chloride, sulphate, nitrate) with the constants given in Table 2;

(ii) for the low–molecular–weight organic components, only the citrate ion was considered (Table 2) as it is present in human serum at a relatively high concentration (1.6×10^{-4} M) and has a relatively high stability constant for Th(IV) complexation [25];

(iii) both HSTf and HSA proteins were considered with the constants given in Figures 2 and 3 captions;

(iv) given the high affinity of iron for HSTf, we can safely consider that trace concentrations of Th(IV) can not compete with Fe(III) for HSTf binding sites. The number of available HSTf binding sites was therefore set to 70 % of the total capacity, corresponding to the fraction of HSTf not loaded with ferric ion [35];

(v) Ca was considered as a competitive metal ion for HSA sorption sites [28].

In agreement with experiments performed with blood serum, 99.9 % of Th(IV) is predicted bound to the protein pool, 99% being attached to HSTf. A less good agreement was obtained using Th(IV)/HSTf constants extrapolated from the relation of Sun et al. [14] reviewed by Ansoborlo et al. [29] (log K_1=19.3 and log K_2=18.2): the model predicted 48 % of Th(IV) under the form $Th(OH)_2(CO_3)_2^{-2}$, the rest being bound to HSTf. This shows that the extrapolation is misleading in the present case resulting in a

underestimation of Th(IV)/HSTf interaction. The values to be used and which can be recalculated from our data according to Eq. (2) are log K_1 =21.2 and log K_2=20.2. All the results show that HSTf is the key component governing Th(IV) speciation in blood serum.

3.3. Potential of DTPA as chelating agent for Th(IV) in blood serum

3.3.1. Theoretical considerations

The DTPA ability to complex Th(VI) under biological conditions was first studied by DUS experiment in the physiological-type model medium (pH=7.4, 0.1 M NaCl) in the presence of HSTf. Complexation of Th(IV) with citrate was studied for comparison. The results of the titration curves are given in Figure 5. Whereas the citrate ion cannot compete with HSTf for Th(IV) in the whole range of ligand concentration studied (10^{-6} M – $3.5.10^{-4}$ M) (see Th(IV)–citrate constant in Table 2 and complexation constants previously determined with HSTf), DTPA starts to complex Th(IV) at the concentration of 10^{-6} M to reach conditions where 100% of Th(IV) is complexed with DTPA. An attempt of quantification was also done for the DTPA series. Since the recent NEA-OECD database does not propose any stability constant for Th/DTPA interaction, we have used two different quantitative models. The first one involves one complex ThL^- with log K of 33.1 (I=0) [17] whereas the second one considers different species proposed in the NIST database [18], i.e. ThL^-, ThHL and $ThOHL^{2-}$ with thermodynamic constants recalculated at I=0 (Table 2). The ThL^- is the major species under studied experimental conditions. A good agreement between the experiment and the calculation was obtained for logK (ThL^-) > 10^{29}. This is in agreement with published constants and this allows an indirect validation of the complexation constants quantifying the interaction between Th(IV) and HSTf. We will next use the data from [17] for the simulations.

DTPA is a stronger chelating agent than citrate and its strong affinity for Th(IV) explains the application of this ligand as a decorporation agent [16]. Note that hydroxypyridone ligands appear more efficient than DTPA for *in vivo* decorporation of Th(IV) [4, 5]. In such an application, the amount of chelating agent is relatively important allowing to reach high concentrations at the injection points (about 0.01 M after injection of 200 mL of saline buffer considering a common dose of 30 µmol/kg body mass and a 70 kg person) and after dilution in the blood (~ 4.10^{-4} M for 5 L of blood volume) [36]. According to our simple model developed for the serum, the efficiency of complexation is predicted *in vivo* in both conditions with 100 % of Th(IV) bound to DTPA considering trace concentrations of Th(IV) (10^{-9} M). Taking into account the amount of ligand injected, we have neglected competition effects with trace metal ions but we have considered a possible competition with major cations using the DTPA/M^{2+} (M^{2+}=Mg^{2+}, Fe^{2+}, Ca^{2+}) constants and "free" blood concentrations (i.e. not bound to proteins) given in [18], and [29], respectively. The complexation with Th(IV) can thus occur and the complex can be eliminated naturally when used immediately after the contamination.

The quantitative analysis show that the complexation becomes inefficient (~ 1% complexed) for a DTPA concentration of $\leq 10^{-5}$ M. It is worth saying that this inefficiency is not related to the HSTf *vs* DTPA competition but to the increasing competition with the divalent Fe^{2+}, Mg^{2+} and Ca^{2+} ions as the ligand concentration decreases. When the Th(IV)/M(II) competition was not considered, the complexation remained efficient for trace DTPA concentration (10^{-9} M) with 71 % of Th(IV) complexed with DTPA. For TAT application, the concentration of injected chelate is usually about 10^{-9} M reaching a value around 5.10^{-11} M after dilution in the blood serum. Taking into account this concentration, DTPA will be inefficient to complex Th(IV) *in vivo*.

3.3.1. In vitro studies

In vitro experiments have been carried out to assess the reliability of the above predictions. Th(IV) speciation was followed by ultrafiltration allowing the separation between low-molecular (i.e. DTPA) and high-molecular (i.e. HSTf) weight compounds. Considering the sensitivity of the ICP-MS and the dilution required for the analysis, Th and DTPA concentrations were fixed at 5.10^{-7} M and 10^{-6} M, respectively. The concentrations are weak enough to expect an inefficient Th(IV) complexation (see the above section).

Experimental results are shown in Figure 6. In the first experiment, Th(IV) is pre-equilibrated with serum before addition of DTPA. In agreement with the prediction, DTPA cannot catch Th(IV) in the blood serum. In the second experiment, DTPA was pre–equilibrated with Th(IV) in 0.1 M NaCl before addition in the human serum. The Th–DTPA complex appears surprisingly stable. This is explained by the strong kinetic stability of Th–DTPA complex: the reaction time is not sufficient to reach equilibrium conditions in the time scale studied (till five hours). Five hours correspond to about 10 half-lives of ^{226}Th, the time for the radionuclide to "disappear". Therefore, although DTPA cannot complex Th(IV) *in vivo*, it may be efficient to carry the radionuclide in TAT, when the biological molecule is being labelled before injection.

4. Conclusion

HSTf governs Th(IV) speciation in the blood serum. This was shown both experimentally and theoretically. Using the generally used Langmuir–type model, values of $10^{21.2}$ and $10^{20.2}$ were obtained for strong and weak sites, respectively. Although DTPA appears to be a good decorporation agent, the thermodynamic predicts that its concentration used in TAT is not sufficient to allow an efficient complexation of Th(IV). This inefficiency is surprisingly not related to the competition with HSTf but is related to the strong competition with major

divalent metal ions for DTPA. However, the high kinetic stability of Th–DTPA complex allows use of the ligand for synthesis of ^{226}Th labeled biomolecules, when the complex is formed before injection. Studies on the stability and cytotoxicity of ^{226}Th–DTPA–antibody contructs *in vitro* and *in vivo* are ongoing.

Table of Abbreviations

DTPA	diethylene triamine pentaacetic acid
HSTf	human serum transferrin
HSA	human serum albumin
TAT	targeted alpha therapy
DUS	difference ultraviolet spectroscopy
HPAEC	high-pressure-anion exchange chromatography
NTA	nitrilotriacetic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ICP-MS	inductively conducted plasma - mass spectrometry

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5. Acknowledgment

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Table 1: Experimental conditions

Method	[Th(IV)] (M)	[HSTf] (M)	carbonate	Experiment	Figure
DUS	2.10^{-5}	10^{-5}	atmosphere	NTA titration	1(B)
	2.10^{-6}	10^{-6}	atmosphere	phosphate titration	not shown
	2.10^{-5}	10^{-5}	0 - 0,05 M	carbonate titration	2(B)
	10^{-6} - $3,5.10^{-5}$	10^{-5}	$p(CO_2)$ 1%	Th(IV) titration	2(D)
	2.10^{-5}	10^{-5}	$p(CO_2)$ 1%	competition with HSA (5.10^{-5}-10^{-4} M)	3
	2.10^{-5}	10^{-5}	$p(CO_2)$ 1%	competition with DTPA ($7,5.10^{-7}$-10^{-4} M)	5
	2.10^{-5}	10^{-5}	$p(CO_2)$ 1%	competition with citrate ($7,5.10^{-7}$-5.10^{-4} M)	5
HPEAC	2.10^{-5}	10^{-5}	$p(CO_2)$ 1%	Th(IV)-HSTf interaction	4(A,D)
	2.10^{-5}	-	$p(CO_2)$ 1%	Th(IV)-HSA (6.10^{-5} M) interaction	4(A,D)
	2.10^{-5}	10^{-5}	$p(CO_2)$ 1%	competition with HSA (6.10^{-5} M)	4(B,E)
	2.10^{-5}	-	$p(CO_2)$ 1%	Th(IV)-serum interaction	4(C,F)
ultra-filtration	2.10^{-5}	10^{-5}	$p(CO_2)$ 1%	Th(IV) speciation in serum	not shown
	2.10^{-7}	-	$p(CO_2)$ 1%	*in vitro* studies in human serum with DTPA	6

Table 2: Formation of complexes between Th(IV) and low–molecular–weight ligands; equillibria relevant in the experimental conditions of this work and associated constants used for the modelling. Acid-base properties of the ligands can be found in the references. All constants are given at an ionic strength of I=0, 25°C. The values in italic are recalculated using the truncated Davies equation [26].

	species	**equilibrium reaction**	**logK**	**Ref**
inorganic ligands	$Th(OH)_4$	$Th^{4+} + 4\ OH^- = Th(OH)_4$	-17.4	[24]
	$(Th)_4(OH)_{12}^{+4}$	$4Th^{4+} + 12H_2O = (Th)_4(OH)_{12}^{+4} + 12H^+$	-26.6	[24]
	$Th(OH)_2(CO_3)_2^{-2}$	$Th^{4+} + 2OH^- + 2\ CO_3^{2-} = Th(OH)_2(CO_3)_2^{-2}$	36.8	[24]
	$Th(H_2PO_4)_2^{+2}$	$Th^{+4} + 2H_3PO_4 = Th(H_2PO_4)_2^{+2} + 2H^+$	6.2	[24]
	$ThSO_4^{+2}$	$Th^{+4} + SO_4^{-2} = ThSO_4^{+2}$	6.2	[24]
	$ThCl^{+3}$	$ThCl^{+3}$	1.7	[24]
	$Th(NO_3)^{+3}$	$Th^{+4} + NO_3^- = ThNO_3^{+3}$	1.3	[24]
citrate ion	ThL^+	$Th^{4+} + L^{-3} = ThL^+$	15.7	[25]
DTPA	ThL^-	$Th^{+4} + L^{-5} = ThL^-$	*32.9*	[18]
	ThLH	$ThL^- + H^+ = ThLH$	*2.37*	[18]
	$ThLOH^{-2}$	$Th^{+4} + L^{-5} + H_2O = ThLOH^{-2} + H^+$	*23.6*	[18]
	ThL^+	$Th^{+4} + L^{-5} = ThL^-$	33.1	[17]
NTA	ThL^+	$Th^{+4} + L^{-3} = ThL^+$	17.1	[26]
	ThL_2^{-2}	$ThL^+ + L^{-3} = ThL_2^{-2}$	14.2	[26]

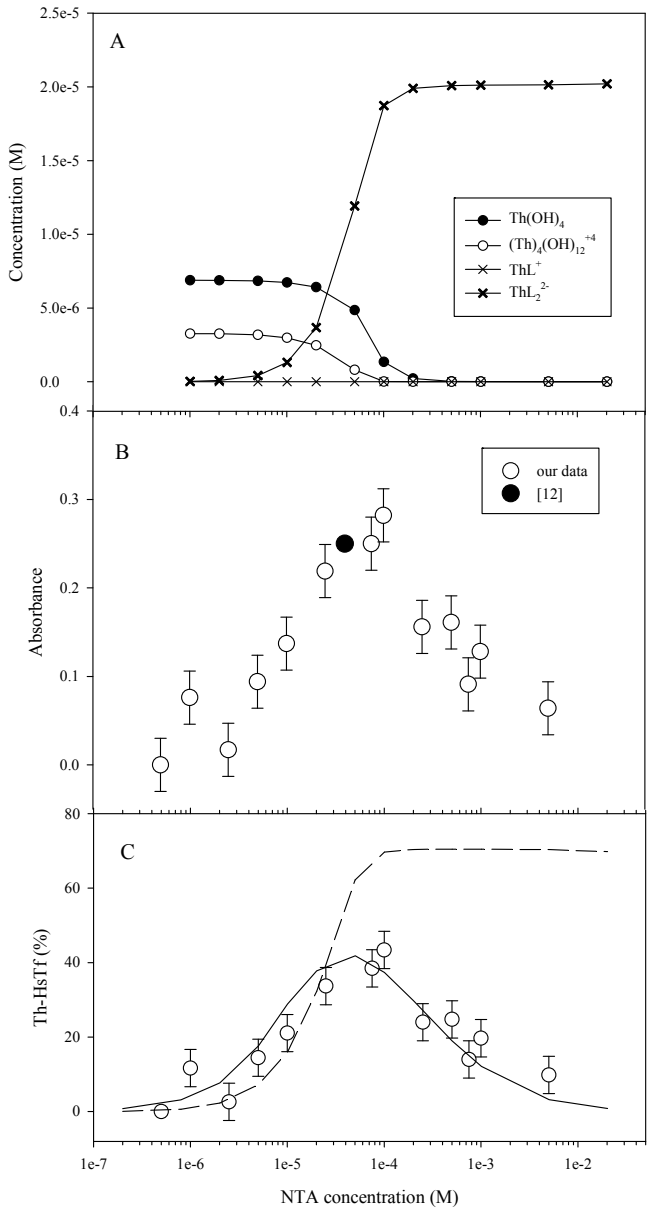

Figure 1: Complexation of Th(IV) as a function of NTA concentration. Experimental conditions and parameters used for the modelling with low–molecular weight compounds are summarized in Tables 1 and 2, respectively. (A) Th(IV) speciation calculated in the absence of HSTf. (B) Absorbance measured as a function of NTA concentration and comparison with literature data [12]. (C) Comparison between experimental data and modelling considering one NTA (solid line; log $K_1 = 31$, log $K_2 = 30$) and two NTA (dotted line; log $K_1 = 41$, log $K_2 = 40$) acting as synergistic anions. L^{3-} stands for deprotonated NTA.

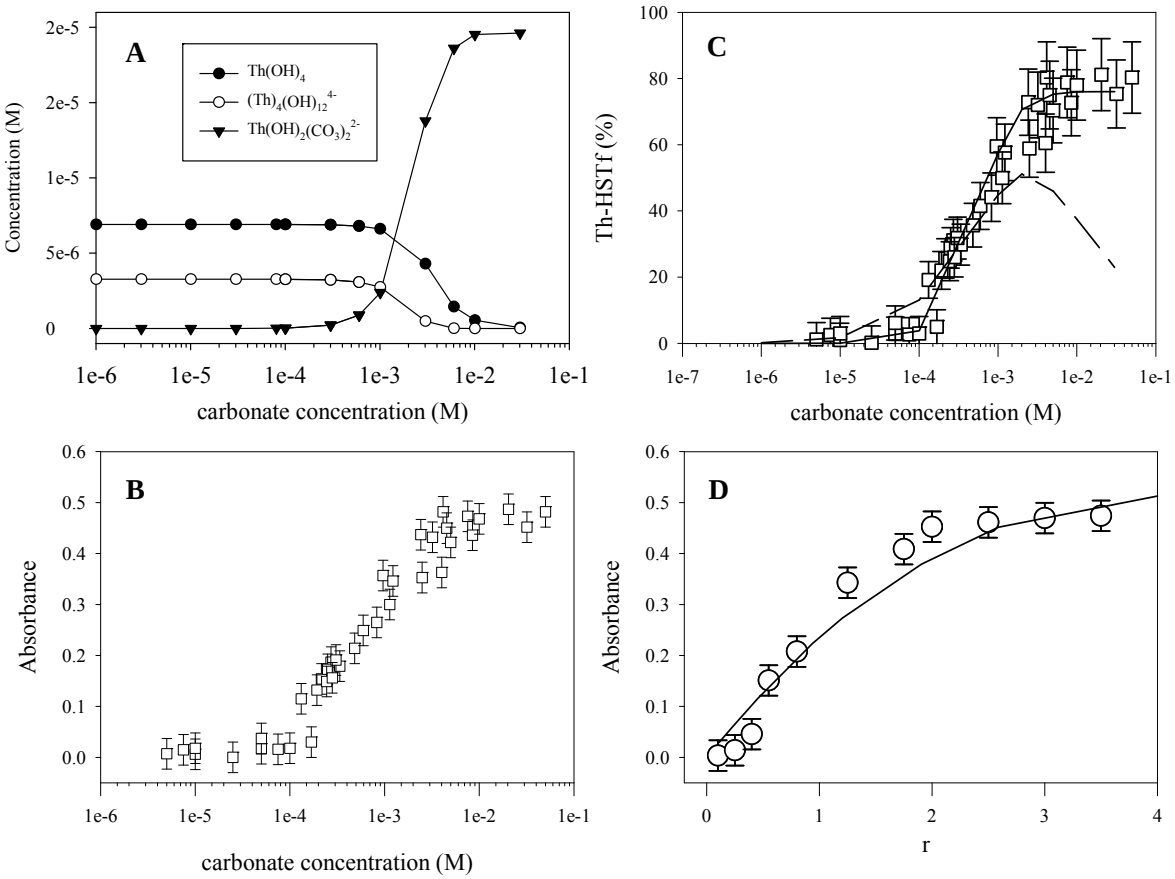

Figure 2: Complexation of Th(IV) by HSTf in the presence of carbonates. Experimental conditions and parameters used for the modelling with low–molecular–weight compounds are summarized in Tables I and II, respectively. (A) Th(IV) speciation calculated in the absence of HSTf. (B) Absorbance measured as a function of carbonate concentration. (C) Comparison between experimental data and modelling considering one carbonate (dotted line; log $K_1 = 27$, log $K_2 = 26$) and two carbonates (solid line; log $K_1 = 33.5$, log $K_2 = 32.5$) acting as synergistic anions. (D) Absorbance measured as a function of the ratio (r) between total Th and HSTf concentrations. The line represents the simulation made with log $K_1 = 33.5$, log $K_2 = 32.5$ and $(\Delta\varepsilon_{obs}) = 60 \times 10^3$ M^{-1} cm^{-1}.

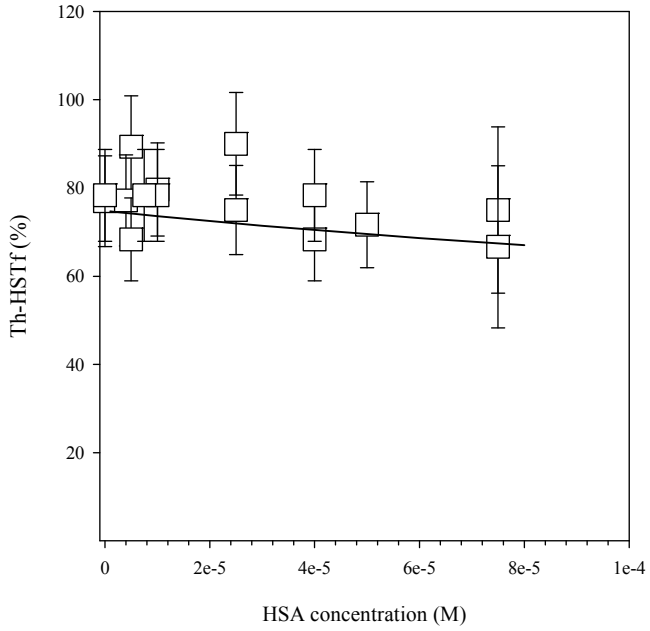

Figure 3: Competition between HSTf and HSA followed by DUS. Experimental conditions and parameters used for the modelling with low-molecular weight compounds are summarized in Tables 1 and 2, respectively. Th(IV)/HSTf interaction was described considering two carbonates bound with log K_1 = 33.5 and log K_2 = 32.5. Th(IV)/HSA interaction was described using a Langmuir–type model with $K = 10^{17.5}$.

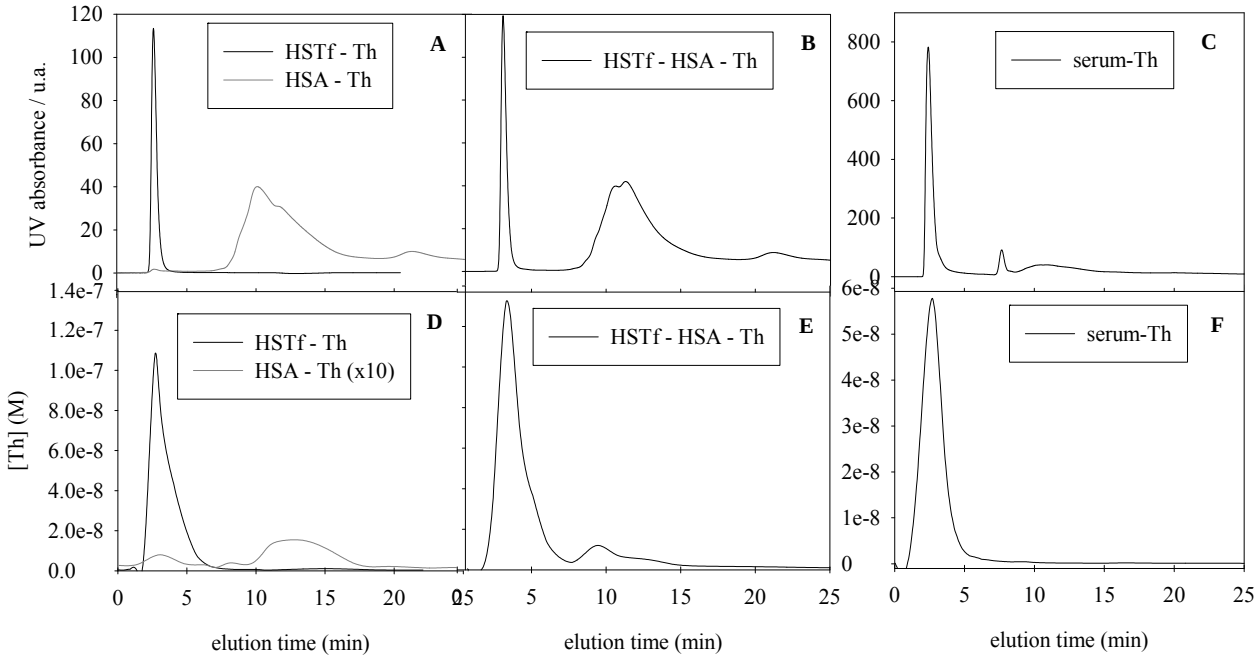

Figure 4: UV– (A,B,C) and ICP–MS– (D,E,F) chromatograms determined by HPAEC. See Table 1 for the experimental conditions. Eluent = 0.1 M NaCl, 2.10^{-3} M PBS, pH 7.4, 1% CO_2.

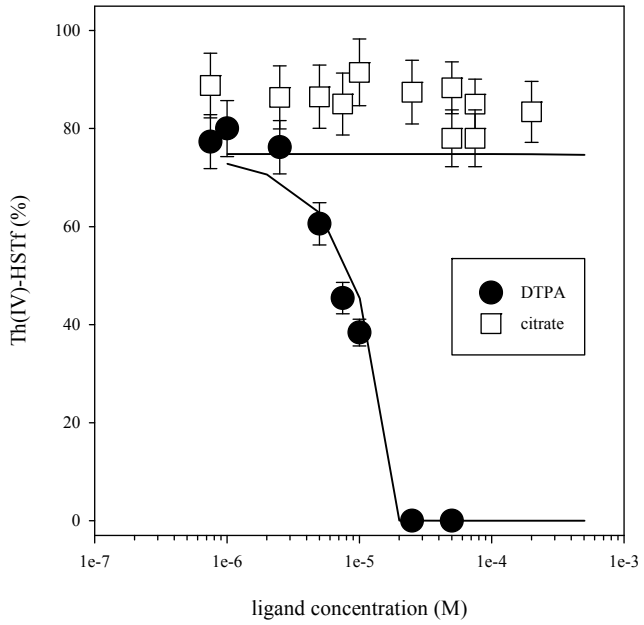

Figure 5: Competition between HSTf and DTPA and citrate followed by DUS. Experimental conditions and parameters used for the modelling with low–molecular–weight compounds are summarized in Tables 1 and 2, respectively. Th(IV)/HSTf interaction was described considering two carbonates bound with log K_1 = 33.5 and log K_2 = 32.5.

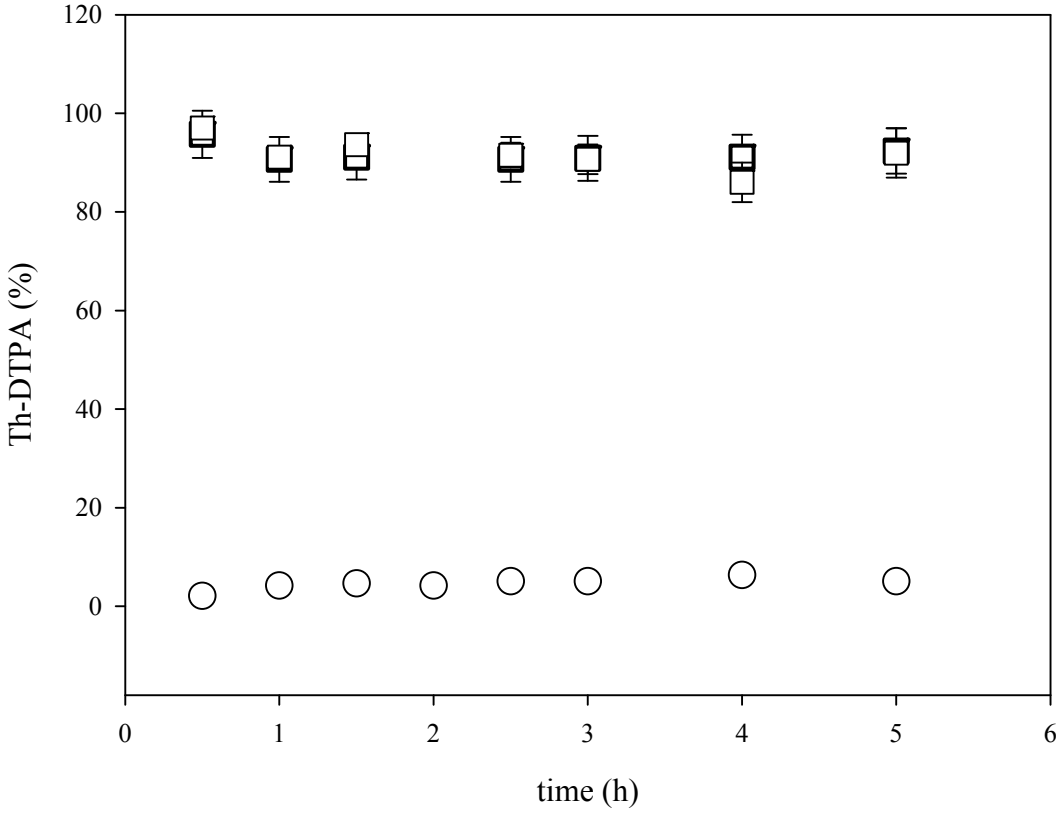

Figure 6: Stability of Th–DTPA complex in the serum followed by ultrafiltration as a function of the time. DTPA and Th concentration were fixed at 10^{-6} and 5.10^{-7} M, respectively. (□) Th–DTPA was pre–formed before addition in the serum. (O) Th was added in the serum containing DTPA.