Radiochim. Acta **95**, 329–334 (2007) / **DOI** 10.1524/ract.2007.95.6.329

Radiochemical and biological behaviour of ^{153}Sm and ^{166}Ho complexes anchored by a novel bis(methylphosphonate) tetraazamacrocycle

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(Received June 1, 2006; accepted in revised form January 2, 2007)

Radiolanthanides / Mixed-tetraazamacrocycles / Radiopharmaceuticals / Biodistribution

Summary. The novel bis(methylphosphonate) 2,2′-[4,10-bis-(phosphonomethyl)-1,4,7,10-tetraazacyclododecane-1,7-diyl] diacetic acid, *trans*-H_6DO2A2P, has been synthesized and characterized by multinuclear NMR spectroscopy (^{1}H, ^{13}C and ^{31}P). ^{153}Sm and ^{166}Ho complexes with *trans*-H_6DO2A2P have been prepared in high yield using a 1 : 2 metal to ligand molar ratio at 70 °C, pH 8–9. These complexes are hydrophilic, negatively charged and stable *in vitro* under physiological solutions, up to 48 h. They present a low plasmatic protein binding and some *in vitro* hydroxyapatite adsorption, mainly for ^{166}Ho-*trans*-DO2A2P. Both complexes are stable *in vivo*, have a fast tissue clearance from most organs and a rapid total excretion from whole animal body. Moderate bone uptake was also observed but the accumulated radioactivity rapidly decreases with time.

Introduction

It is well known that tetraazamacrocyclic ligands, due to their cyclic and preorganized nature, can encapsulate different metal ions, especially lanthanides, forming complexes with enhanced thermodynamic stability and kinetic inertness, when compared with analogous acyclic ligands [1, 2]. In the last two decades, the chemistry of lanthanide complexes based on DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) and on DOTP ([1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrayl tetra(methylene)] tetra phosphonic acid) was largely studied, including the evaluation of their potential for biomedical applications, such as magnetic resonance imaging (MRI), or molecular imaging and/or therapy in nuclear medicine [3–5]. For MRI, the majority of the approved contrast agents are based on Gd^{3+} complexes, due to the high effective magnetic moment and relatively long electron spin relaxation time of the Gd(III) ion. $[Gd\text{-}DOTA]^-$ is one of the most effective and safe contrast agents available, but is nonspecific, *i.e.* distributing throughout all extracellular space, and a relatively high dosage of such agent is required for significant MRI contrast enhancement. However, lower doses could be employed if the contrast agents were delivered to a specific organ, and this justifies the interest on finding more specific contrast agents [4]. Biodistribution studies have shown that ^{159}Gd-DOTP binds significantly to bone, which could improve MRI contrast [6]. However, hydroxyapatite binding assays revealed that this complex is MRI silent when bound to bone [7]. Recently, Lukes *et al.* have explored the chemical properties of some DOTA-like lanthanide complexes, in which one acetate arm has been replaced by a phosphorous acid moiety, demonstrating their interest for the design of MRI contrast agents [8–10]. Complexes of Gd^{3+} with cyclen derivatives containing two acetates and two ethyl methylenephosphonate or methyleneethylphosphinate groups were also evaluated. Despite their lower thermodynamic stability relative to the parent complex Gd-DOTA, the data obtained for this family of complexes have shown that their structure and dynamic behaviour in solution could be very useful in some MRI applications [11].

In spite of the excellent nuclear properties of the radiolanthanides for therapeutic applications, namely ^{153}Sm, ^{166}Ho and ^{177}Lu, there is only one complex, ^{153}Sm-EDTMP (1,2 diaminoethane-N,N,N′,N′-tetra(methylenephosphonic acid)), in clinical use for bone pain palliation [12, 13]. However, this radiopharmaceutical is not kinetically inert and a large excess of ligand is necessary for its preparation, due to the acyclic nature of EDTMP [12]. This justifies the interest on radiolanthanide complexes with tetraazamacrocycles [2]. Complexes anchored by macrocycles with methylphosphonate pendant arms, such as ^{153}Sm/^{177}Lu-DOTP and ^{166}Ho-DOTP, have shown efficacy in bone pain palliation and bone marrow ablation in multiple myeloma patients, respectively [14–18].

As part of our on-going research work on lanthanide complexes with 13- and 14-membered tetraazamacrocycles with methylcarboxylate and/or methylphosphonate pendant arms, we have shown recently that ^{153}Sm/^{166}Ho-TRITP ([1,4,7,10-tetraazacyclotridecane-1,4,7,10-tetrayl tetra(methylene)] tetra phosphonic acid) have considerable bone uptake and a promising biological profile. However, the relatively slow rate of formation of the complexes, the low

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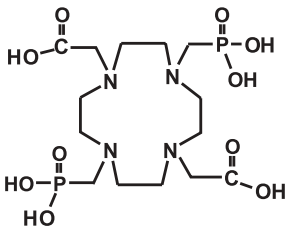


***trans*-H_6DO2A2P**

Fig. 1. Structure of the macrocyclic ligand 2,2′-[4,10-bis(phosphonomethyl)-1,4,7,10-tetraazacyclododecane-1,7-diyl] diacetic acid (*trans*-H_6DO2A2P).

selectivity for a specific metal ion and the high osmolarity of these complexes, due to their high charge, may be disadvantageous [19]. Aiming to overcome some of these problems, we have focused attention on the synthesis and characterization, by multinuclear NMR spectroscopy, of the novel macrocycle 2,2′-[4,10-bis(phosphonomethyl)-1,4,7,10-tetraazacyclododecane-1,7-diyl] diacetic acid, *trans*-H_6DO2A2P (Fig. 1) and evaluated its coordination capabilities towards the radiolanthanides ^{153}Sm and ^{166}Ho. We also report on the *in vitro* and *in vivo* biological behaviour of radiolanthanide complexes ^{153}Sm/^{166}Ho-*trans*-DO2A2P.

Experimental section

Reagents

Chemicals and solvents were of reagent grade and were used without further purification, unless stated otherwise. 1,4,7,10-tetraazacyclododecane (cyclen) was purchased from Strem (New-buryport, Mass, USA). Paraformaldehyde, triethylphosphite, diethyl ether, acetonitrile, benzene, silica gel (200–400 mesh), calcium phosphate dibasic (hydroxyapatite), D_2O, $CDCl_3$ and all other standard reagents were obtained from Aldrich Chemical Co.

Enriched Sm_2O_3 (98.4% ^{152}Sm) was obtained from Campro Scientific and natural Ho_2O_3 (99.9%) from Strem Chemicals. All materials used for radiochemical and biological evaluation of radioactive compounds were reagent grade unless otherwise specified.

Analytical methods

^{1}H (300 MHz), ^{13}C (75.5 MHz) and ^{31}P (121.5 MHz) NMR spectra were recorded on a Varian Unit Inova-300 spectrometer. ^{1}H spectra were recorded in $CDCl_3$ or in D_2O. ^{13}C NMR spectra were recorded in $CDCl_3$ (*versus* $CDCl_3$ (77 ppm)) or D_2O (*versus* CH_3 of tert-butyl alcohol (31.2 ppm)). ^{31}P NMR spectra were recorded in $CDCl_3$ or in D_2O, and were referenced to external 85% aqueous H_3PO_4 solution ($\delta = 0$ ppm).

Production of ^{153}Sm and ^{166}Ho

^{153}Sm ($T_{1/2} = 46.8$ h; $\beta_{max} = 0.67$ MeV, 34%; 0.71 MeV, 44%; 0.81 MeV, 21%; $\gamma = 0.103$ MeV, 38%) and ^{166}Ho ($T_{1/2} = 26.8$ h; $\beta_{max} = 1.85$ MeV, 51%; 1.77 MeV, 48%; $\gamma =$ 80.6 keV, 7.5%; 1.38 MeV, 0.90%) were produced in the ITN Portuguese Research Reactor (RPI) by thermal neutron bombardment of isotopically enriched $^{152}Sm(NO_3)_3$ or natural $Ho(NO_3)_3$, respectively, as previously described [21]. The specific activities of the radionuclides, after 3 h irradiation and at EOB, were 110–150 MBq/mg for ^{153}Sm and 220–260 MBq/mg for ^{166}Ho. The radionuclidic purity of the ^{153}Sm and ^{166}Ho solutions was assessed by γ-ray spectrometry using a Ge (Li) detector coupled to an Accuspec B Canberra multichannel analyzer [19]. The spectra were processed, following efficiency calibration with a ^{152}Eu source [21]. The ^{153}Sm and ^{166}Ho activities produced after irradiation were measured in a dose calibrator (Aloka Curiemeter IGC-3).

Synthesis

The derivative 1,7-bis(acetic acid tert-butyl ester)-1,4,7,10-tetraazacyclododecane was synthesized according to the procedure described in the literature [20].

1,7-bis(diethylphosphoryl)methyl-4,10-bis(carboxymethyl ethyl ester)-1,4,7,10-tetraazacyclododecane (1)

550 mg (1.37 mmol) of 1,7-bis(acetic acid tert-butyl ester)-1,4,7,10-tetraazacyclododecane and 1.57 g (9.47 mmol) of triethylphosphite were dissolved in 20 ml of dry benzene, and the resulting solution refluxed. 330 mg (10.98 mmol) of dry paraformaldehyde was slowly dropped into the refluxing solution, over a period of 4 h. The mixture was refluxed overnight. The solvent was removed under vacuum and the clear pale yellow oil kept under high vacuum at 40–50 °C for 12 h, to remove volatile impurities. The resulting oil was purified by column chromatography on silica gel (EtOH: 25% aq. NH_3). The fractions containing **1** were collected, dried under vacuum and the resulting pale yellow oil was analysed. Yield: 403 mg (42%). ^{1}H NMR ($CDCl_3$), δ (ppm): 3.99 (q, 8H, P(O)OCH_2CH$_3$, $^3J_{HH} = {}^3J_{PH} = 7.5$ Hz), 2.89–277 (br, m, 24H, cyclen-CH_2-N and NCH_2COOtBu and CH_2P), 1.25 (t, 18H, CH_3 t-Bu), 1.89 (t, 12H, P(O)OCH$_2$$CH_3$). ^{13}C NMR ($CDCl_3$/TMS), δ (ppm): 170.0 (*C*OOt-Bu), 81.0 (*C*(CH$_3$)$_3$), 61.6 (P(O)OCH_2CH$_3$, $^2J_{PC} = 3$ Hz), 55.8 (CH_2COOt-Bu), 52.3 (NCH_2CH_2N), 51.9 (NCH_2CH_2N and CH_2(P(O)OCH$_2$CH$_3$)), 27.9 (C(CH_3)$_3$), 16.3 (P(O)OCH$_2$$CH_3$). ^{31}P NMR ($CDCl_3$), δ (ppm): 26.8.

2,2′-[4,10-bis(phosphonomethyl)-1,4,7,10-tetraazacyclododecane-1,7-diyl] diacetic acid (*trans*-H_6DO2A2P) (2)

1 (400 mg, 0.57 mmol) was dissolved in 20% HCl (50 ml) and the mixture was refluxed for 24 h. After removing the HCl under vacuum, a clear oil was obtained. This oil was dissolved in H_2O and vacuum dried. After repeating this procedure 3 times a white powder was obtained. Yield: 238 mg (90%). ^{1}H NMR (D_2O/TMS), δ (ppm): 3.55 (s, 4H, NCH_2COOH), 3.43–3.38 (br, m, 8H, NCH_2CH_2N), 3.27 (d, 4H, NCH_2P(O)(OH)$_2$, $^2J_{PH} = 12$ Hz), 3.06 (br, m, 8H, NCH_2CH_2N). ^{13}C NMR (D2O/tert-butyl alcohol), δ (ppm): 176.3 (COOH), 56.2 (CH_2COOH), 54.7 (NCH_2CH_2N) 54.5 (CH_2P(O)(OH)$_2$, $^1J_{PC} = 4$ Hz), 51.4 (NCH_2CH_2N). ^{31}P NMR (D_2O/KOD, pD = 10), δ (ppm): 20.5.

Synthesis of ^{153}Sm and ^{166}Ho complexes

Radiolabelling of the macrocycle was performed by dissolving the ligand (5 mg) in H_2O (0.3 ml) at 30 mM final concentration, followed by the addition of an adequate amount of ^{153}Sm or ^{166}Ho nitrate solution to achieve a 19 mM ligand concentration (1 : 2 $M : L$ molar ratio). The labelling was optimized by changing the pH (6–10) and the temperature. Labelling efficiency, chelation kinetics and stability of the radiolanthanide complexes were followed by ascending silica gel ITLC (Polygram, Macherey–Nagel) developed with the mobile phase MeOH : H_2O : NH_4OH (2 : 4 : 0.2). In this system the ^{153}Sm/^{166}Ho complexes migrate with $R_f = 1.0$, while $^{153}Sm(NO_3)_3$ and $^{166}Ho(NO_3)_3$ remain at the origin. The colloidal radioactive forms, if present, also remain at the origin. These species can be assessed by ascending instant thin layer chromatography using silica gel ITLC strips developed with saline. In this system, both the radiolanthanide complexes and ^{153}Sm/$^{166}Ho(NO_3)_3$ migrate with $R_f = 1.0$.

In vitro stability studies

The *in vitro* stability of the complexes was evaluated in saline, 0.1 M phosphate buffer (pH 7.4), 0.1 M tris-HCl buffer (pH 7.4), 0.1 M glycine-HCl solution (pH 4.0) and human serum, at 37 °C at various time points (up to five days). Typically, 50 μl of each ^{153}Sm- or ^{166}Ho-complex was added to 100 μl of the different solutions and stored at 37 °C. Daily, an aliquot of each mixture was taken and evaluated by ITLC analysis, as described above. The percentage of the radiochemical impurities was then calculated.

Adsorption studies

Adsorption of the ^{153}Sm and ^{166}Ho/*trans*-DO2A2P complexes onto hydroxyapatite (HA) was determined by incubation with different amounts of HA. Briefly, 50 μl of the complex (3–5 MBq/50 μl; 13.7–14 mM) was incubated by continuous mixing for 1 h at room temperature with 5, 10, 25, 50, 100 and 150 mg of HA in 2 ml of 0.1 M tris-HCl buffer (pH 7.4). Immediately after incubation, the mixture was centrifuged at 10 000 r.p.m. to achieve complete sedimentation, and the liquid phase was separated. The solid phase was washed twice with 2.5 ml of 0.1 M tris-HCl buffer (pH 7.4). Washing buffer was pooled with liquid phase. The radioactivity in the liquid and solid phases was measured in a dose calibrator.

Complex charge, lipophilicity and protein binding

The overall charge of the radioactive complex was determined by electrophoresis in 0.1 M tris-HCl buffer (pH 7.4), as previously described [21].

Lipophilicity was assessed by determination of the partition coefficient (P) *n*-octanol/saline and expressed as log P, according to the previously described method [21].

Plasmatic protein binding was determined by gel filtration on Sephadex G-25 using saline as eluent after 1 h incubation of the radiolanthanide complex (100 μl) with 1 ml of human blood plasma, as previously reported [21].

In vivo studies

Biodistribution studies

The *in vivo* behaviour of the radioactive complexes was evaluated in groups of 4–5 female CD-1 mice (randomly bred, from Charles River Laboratories, Spain) weighing approximately 20–22 g. Animals were intravenously (i.v.) injected through tail vein with 100 μl (10–15 MBq/100 μl) of the radiolanthanide complex solution, maintained on normal diet *ad libitum* and were sacrificed by cervical dislocation at 30 min, 2 h and 24 h post injection, according to a previously described method [19, 21]. Results were expressed as percentage of injected dose per gram of organ (% I.D./g organ + SD). Whole body excretion of the radioactivity was assumed to be the difference between the measured radioactivity in the injected and sacrificed animal and was expressed as percentage of injected dose (% I.D.).

The *in vivo* stability of the complexes was assessed by urine and blood ITLC analysis, using the above referred experimental conditions for the radiochemical purity evaluation.

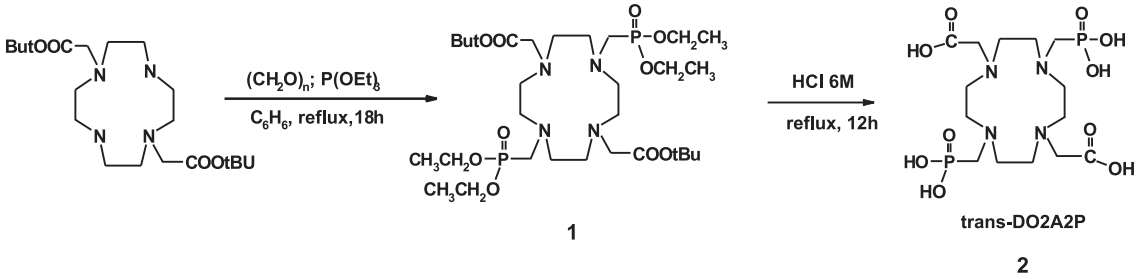
Animal experiments were carried out in accordance with the guidelines on the use of living animal in scientific investigation, and followed the principles of laboratory animal care.

Results and discussion

Synthesis of the novel bis(methylphosphonate) tetraazamacrocycle

The synthesis of the novel macrocyclic ligand 2,2′-[4,10-bis(phosphonomethyl)-1,4,7,10-tetraazacyclododecane-1,7-diyl] diacetic acid, *trans*-H_6DO2A2P, was done by a Mannich type reaction, followed by acidic hydrolysis. 1,7-bis(acetic acid tert-butyl ester)-1,4,7,10-tetraazacyclododecane reacts with triethylphosphite and dried paraformaldehyde in benzene, affording 1,7-bis(diethylphosphoryl)methyl)-4,10-bis(carboxymethyl ethyl ester)-1,4,7,10-tetraazacyclododecane (**1**) in 42% yield (Scheme 1). Acidic hydrolysis of **1** cleaved the ester groups from the phosphonate and acetate pendant arms giving compound **2** in 90% yield, after recrystallization from water/ethanol. The white powder obtained was formulated as 2,2′-[4,10-bis(phosphonomethyl)-1,4,7,10-tetraazacyclododecane-1,7-diyl] diacetic acid, *trans*-H_6DO2A2P, based on multinuclear NMR spectroscopy.

The synthesis of *trans*-H_6DO2A2P was tried using different experimental conditions, namely different alkyl phosphites. However, we found that the yield of the Mannich type reaction decreases significantly when using diethylphosphite instead of triethylphosphite, or when the paraformaldehyde is added too fast. The reaction of DO2A (1,4,7,10- tetraazacyclododecane-1,7-bis(acetic acid)) with phosphorous acid, in aqueous HCl was also tried. In this reaction *trans*-H_6DO2A2P was formed as indicated by ^{1}H and ^{13}C NMR spectroscopy, but was contaminated with some impurities, most probably lactams or other side products derived from intramolecular reactions. The attempts made to purify this mixture have been unsuccessful and



Scheme 1. Synthesis of *trans*-H_6DO2A2P.

trans-H_6DO2A2P could never be properly isolated using this synthetic methodology.

The structure of *trans*-H_6DO2A2P was confirmed by ^{1}H, ^{13}C and ^{31}P NMR spectroscopy. The ^{1}H NMR spectra in D_2O at acidic pD, consist of two resonances integrating for eight protons each assigned to the methylenic protons of the macrocycle backbone, and two other resonances, integrating for four protons each, corresponding to the methylenic protons of the methylacetate and methylphosphonate pendant arms. In the ^{13}C NMR spectra five resonances were observed for the fourteen carbon nuclei. The resonance at 176.3 ppm was assigned to the carbons of the two carboxylate groups, while the resonances at 56.2 and 54.5 ppm are due to the methylenic carbons of the carboxylate and phosphonate pendant arms, respectively. The carbon atoms of the macrocycle backbone appear as two resonances at 54.7 and 51.4 ppm. Under acidic conditions the ^{31}P NMR spectrum presents only one very broad resonance for the two phosphorus nuclei, but this resonance sharpened significantly at basic pD, appearing at 20.5 ppm [22]. The pattern of the ^{1}H, ^{13}C and ^{31}P NMR spectra agrees with the C2 symmetry expected for *trans*-H_6DO2A2P.

Synthesis of ^{153}Sm and ^{166}Ho complexes

^{153}Sm/^{166}Ho-*trans*-DO2A2P complexes were obtained by reacting ^{153}Sm/^{166}Ho$(NO_3)_3$ solutions with *trans*-H_6DO2A2P in a 1 : 2 metal to ligand molar ratio. The experimental conditions were optimized in order to obtain radiolanthanide complexes with high radiochemical purity. The radiocomplexes were obtained quantitatively (> 98%) at pH 8–9 after 2 hours at room temperature or after 30 min at 70 °C. The labeling yield decreases to 85%–90% when the reactions are carried out at 8 > pH > 10. In order to get a better understanding of the kinetics of these reactions and of the nature and stability of the species formed, kinetics and thermodynamic studies in solution of the *trans*-H_6DO2A2P with lanthanides are in progress [22].

Charge, lipophilicity, plasmatic protein binding and *in vitro* stability

Both ^{153}Sm/^{166}Ho complexes are negatively charged, hydrophilic ($\log P = -1.93$ and -1.32, for ^{153}Sm-*trans*-DO2A2P and ^{166}Ho-*trans*-DO2A2P, respectively) and present a low binding to plasmatic proteins, (1.4% and 1.5% for ^{153}Sm-*trans*-DO2A2P and ^{166}Ho-*trans*-DO2A2P, respectively). These complexes are stable up to five days at 37 °C under physiological conditions, namely in saline, phosphate buffer (pH 7.4) and 0.1 M glycine-HCl solution. But the complexes are stable in human serum up to 2 days. In 0.1 M tris-HCl buffer (pH 7.4), the solution used for the HA binding studies and for electrophoresis, both complexes have proven to be stable for at least 2 h.

Adsorption studies

The binding of ^{153}Sm/^{166}Ho-*trans*-DO2A2P onto HA, the main mineral component of bone, was studied, trying to anticipate the potential of these complexes as bone-seeking agents *in vivo*. Fig. 2 shows the binding to hydroxyapatite of ^{153}Sm-*trans*-DO2A2P and ^{166}Ho-*trans*-DO2A2P as a function of the amount of HA. Analysis of these curves has clearly demonstrated that the ^{166}Ho-*trans*-DO2A2P binds more significantly to HA than the ^{153}Sm-*trans*-DO2A2P, presenting both lower HA binding than the previously described ^{153}Sm/^{166}Ho-DOTP and ^{153}Sm/^{166}Ho-TRITP complexes [19].

Biodistribution studies

Tissue distribution data of ^{153}Sm/^{166}Ho-DO2A2P for the most relevant organs as a function of time after *i.v.* administration in female CD-1 mice are summarized in Fig. 3. These results indicated a rapid clearance of the injected activity from blood stream with no significant uptake in any of the major organs except in kidneys, which is in agreement with the relatively low plasmatic protein binding found in the *in vitro* assays.

The biological profile of both complexes is very similar to that found for ^{153}Sm/^{166}Ho-DOTA in the same animal

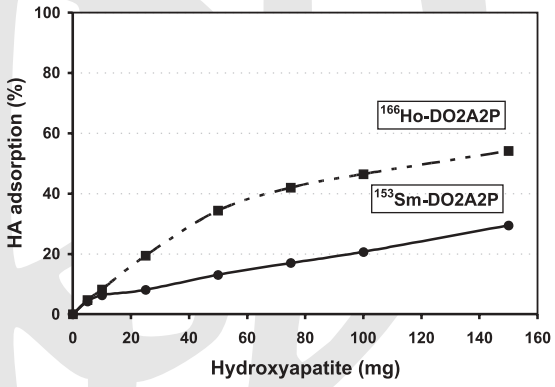


Fig. 2. Hydroxyapatite adsorption of ^{153}Sm/^{166}Ho-DO2A2P *versus* hydroxyapatite amount.

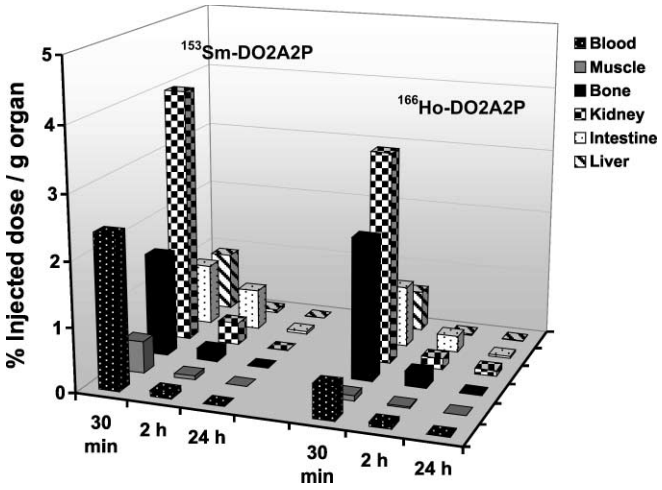


Fig. 3. Biodistribution data (% I.D/g organ) for ^{153}Sm/^{166}Ho-DO2A2P, at 30 min, 2 h and 24 h after *i.v.* administrationin CD-1 mice ($n = 4$–5).

model [19]. The main difference is related to the degree of activity taken by the bone. In fact, a moderate bone uptake, significantly superior to that found for DOTA, was observed. Even so, the retained radioactivity rapidly decreases with time. The bone uptake found for ^{166}Ho-*trans*-DO2A2P at 30 minutes agrees with the *in vitro* results but the fast decrease observed *in vivo* does not make this compound promising for bone pain palliation, when compared with other complexes previously reported, which have shown a significant bone uptake (% ID/g) at 2 h p.i., in the same animal model: 5.5%, ^{166}Ho-TRITP; 5.6% ^{153}Sm-DOTP; 7.6%, ^{166}Ho-DOTP [19].

Whole animal body radioactivity excretion over time is graphically represented in Fig. 4. For both complexes, more than 93% of the radioactivity is excreted *via* urinary pathway, at 2 h after injection. This behaviour correlates well with the high hydrophilic character of the radioactive complexes.

Chromatographic analysis of urine samples collected at different sacrifice times indicated a high *in vivo* stability for these complexes as no metabolites could be identified.

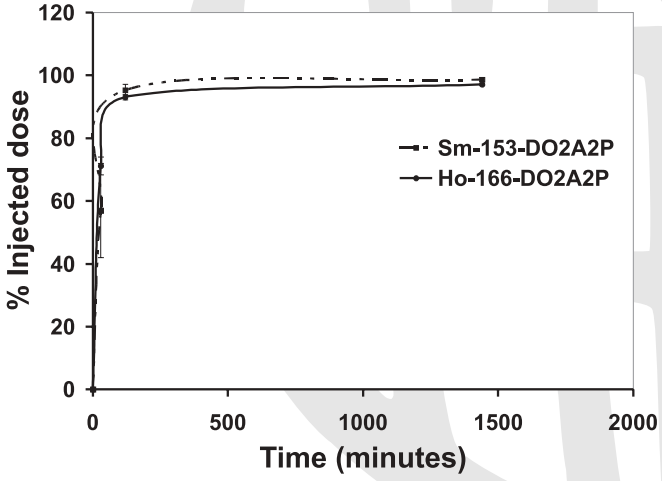


Fig. 4. Excretion data (% I.D.) for ^{153}Sm/^{166}Ho-*trans*-DO2A2P, at 30 min, 2 h and 24 h after *i.v.* administrationin CD-1 mice ($n = 4$–5).

Concluding remarks

A novel 12-membered tetraazamacrocycle containing two acetate and two methylphosphonate pendant arms, *trans*-H_6DO2A2P, was prepared and characterized by ^{1}H, ^{13}C and ^{31}P NMR spectroscopy. ^{153}Sm/^{166}Ho-*trans*-DO2A2P complexes have also been prepared quantitatively using a 1 : 2 metal to ligand molar ratio, at pH 8–9. *In vitro* studies have shown that ^{153}Sm/^{166}Ho-*trans*-DO2A2P complexes are hydrophilic, negatively charged, and stable under physiological conditions, presenting a low plasmatic protein binding. A moderate *in vitro* HA adsorption was found for ^{166}Ho-*trans*-DO2A2P, while ^{153}Sm-*trans*-DO2A2P binds in a lowest degree. The biological profile of these complexes is very similar to the profile obtained for the corresponding DOTA complexes, in the same animal model, the main difference being the degree of activity taken by bone. However, the radioactivity decreases rapidly, which does not make these complexes promising for bone pain palliation. The high *in vivo* stability of ^{153}Sm/^{166}Ho-*trans*-DO2A2P and their biological profile highlight the interest in the synthesis of novel macrocycle ligands with the same backbone but with a different number of phosphonate pendant arms or the replacement of the methylphosphonates by bone resorption inhibitors.

Acknowledgment. The authors acknowledge the financial support from Fundação para a Ciência e Tecnologia (FCT) and POCTI, with co- participation of the European Community Fund FEDER (POCTI/2000/CBO/35859). S. Lacerda thanks FCT for the PhD grant (SFRH/BD/19168/2004). COST ACTION D18 is also acknowledged. The authors thank the ITN Research Portuguese Reactor Group for their help in carrying out the production of ^{153}Sm and ^{166}Ho.

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