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Journal of Inorganic Biochemistry 100 (2006) 270–280
JOURNAL OF Inorganic Biochemistry
www.elsevier.com/locate/jinorgbio

13- and 14-membered macrocyclic ligands containing methylcarboxylate or methylphosphonate pendant arms: Chemical and biological evaluation of their ^{153}Sm and ^{166}Ho complexes as potential agents for therapy or bone pain palliation

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Received 30 June 2005; received in revised form 17 November 2005; accepted 18 November 2005
Available online 4 January 2006

Abstract

The stability constants of La^{3+}, Sm^{3+} and Ho^{3+} complexes with 13- and 14-membered macrocycles having methylcarboxylate (trita and teta) or methylphosphonate (tritp and tetp) arms were determined. All the ligands were labelled with ^{153}Sm and ^{166}Ho in order to evaluate the effect of the macrocyclic cavity size and type of appended arms on their in vitro and in vivo behaviour. The radiolabelling efficiency was found to be higher than 98% for all the complexes, except for those of tetp. All radiocomplexes studied are hydrophilic with an overall negative charge and low plasmatic protein binding. Good in vitro stability in physiological media and human serum was found for all complexes, except the ^{153}Sm/^{166}Ho–teta, which are unstable in phosphate buffer (pH 7.4). In vitro hydroxyapatite (HA) adsorption studies indicated that ^{153}Sm/^{166}Ho–tritp complexes bind to HA having the ^{166}Ho complex the highest degree of adsorption (>80%, 10 mg). Biodistribution studies in mice demonstrated that ^{153}Sm/^{166}Ho–trita complexes have a fast tissue clearance with more than 95% of the injected activity excreted after 2 h, value that is comparable to the corresponding dota complexes. In contrast, the ^{153}Sm–teta complex has a significantly lower total excretion. ^{153}Sm/^{166}Ho–tritp complexes are retained by the bone, particularly ^{166}Ho–tritp that has 5–6% (% I.D./g) bone uptake and also a high rate of total excretion. Thus, these studies support the potential interest of ^{153}Sm/^{166}Ho–trita complexes for therapy when conjugated to a biomolecule and the potential usefulness of the ^{166}Ho–tritp complex in bone pain palliation.

Keywords: Lanthanides; Macrocycles; Biological studies; Radiopharmacy

1. Introduction

Radionuclide therapy employing radiotherapeutic agents is an important emerging area of oncology [1–3]. To develop effective radiopharmaceuticals for therapy it is essential to choose the appropriate radionuclide and the carrier biomolecule to target selectively the disease site [4–6]. The decay characteristics, the ease of production and a versatile chemistry, are the main features for the choice of a radionuclide. ^{153}Sm and ^{166}Ho are easily produced in a nuclear reactor and owing to their favourable decay characteristics are attractive for therapeutic applications, as well as for imaging [7,8].

Complexes of ^{153}Sm with ligands containing phosphonate groups, such as *Quadramet*™, are in clinical use for bone pain relief and treatment of bone metastasis, while ^{166}Ho complexes with dotp and with dtpa are under clinical

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0162-0134/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.jinorgbio.2005.11.011

trials for myeloablative treatment of multiple myelomas or for intravascular radiation therapy [9–15].

^{153}Sm and ^{166}Ho complexes with tetraazamacrocycles linked to different biomolecules, such as monoclonal antibodies or peptides, have also been considered excellent candidates for therapy and are under investigation [16–24].

For therapeutic proposes in nuclear medicine, the radiotherapeutic agent should be stable in vivo in order to prevent their dissociation in blood and the formation of species resulting from binding to blood components [25]. Structural factors such as the rigidity, the cavity size, the nature and number of donor atoms of the macrocyclic bifunctional chelators play a significant role on the chemical and biological behaviour of their complexes [26–28]. Taking into account these factors and our interests on finding radioactive lanthanide complexes suitable for conjugation to biomolecules and/or for bone pain palliation, we studied a series of 13- to 14-membered tetraazamacrocycles (Fig. 1). The stability constants of the La^{3+}, Sm^{3+} and Ho^{3+} complexes with trita, teta, tritp and tetp have been determined and the ^{153}Sm and ^{166}Ho complexes prepared and evaluated in vitro and in vivo. For comparison, ^{153}Sm– and ^{166}Ho–dota and dotp complexes were also synthesized and their in vivo behaviour evaluated in the same animal model.

2. Experimental

2.1. Materials and methods for evaluation of radionuclidic and radiochemical purity

Enriched Sm_2O_3 (98.4% ^{152}Sm) was obtained from Campro Scientific and natural Ho_2O_3 (99.9%) from Strem Chemicals. All the ligands used (Fig. 1) were synthesized and purified according to reported methods [29,30]. For some of the experiments with radionuclide elements, dota and teta were obtained from Strem Chemicals and Aldrich Chemicals Co. Inc., respectively. Calcium phosphate dibasic (hydroxyapatite) was purchased from Aldrich Chemical Co. Inc. All materials were reagent grade unless otherwise specified. 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 analyser. The spectra were processed, following efficiency calibration with a ^{152}Eu source. The ^{153}Sm and ^{166}Ho activities produced after irradiation were measured by an ionization chamber (Aloka Curiemeter IGC-3).

The radiolabelling efficiency and stability evaluation of the radiocomplexes were accomplished by ascending instant thin layer chromatography using silica gel strips (Polygram, Macherey-Nagel). Radioactive distribution on the ITLC (Instant Thin Layer Chromatography) strips was detected using a Berthold LB 505 γ detector coupled to a radiochromatogram scanner. All solvents used as mobile phase were chromatography grade. The radioactivity from samples of protein binding studies was measured by a γ counter (Berthold LB 2111).

2.2. Potentiometric measurements

2.2.1. Reagents and solutions

Solutions of lanthanide nitrates of analytical grade (0.045–0.050 M) were prepared with demineralized water (from a Millipore/Milli-Q system), kept in excess of HNO_3, and standardized by titration with Na_2H_2 edta (disodium ethylenediamine tetraacetate) [31]. Carbonate free solutions of the titrant, NMe_4OH, were freshly prepared and were discarded when carbonate was about 0.5% of the total amount of base [25]. For back titrations, 0.100 M HNO_3 solution was used.

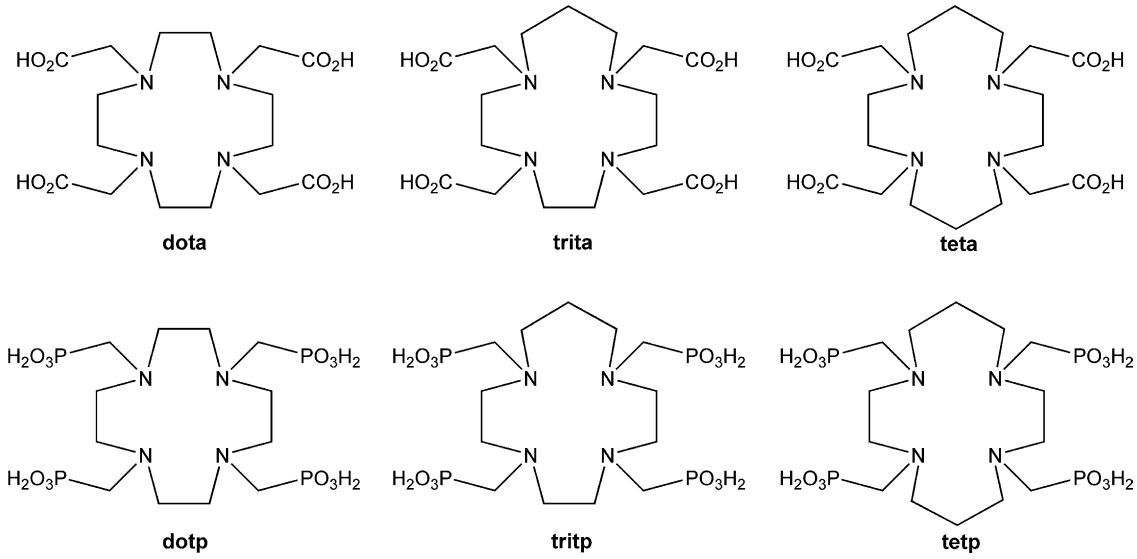

Fig. 1. Structure of the macrocyclic ligands containing methylcarboxylate (dota, trita and teta) and methylphosphonate (dotp, tritp and tetp) pendant arms used in this study.

2.2.2. Equipment and work conditions

An Orion 720A measuring instrument was used together with a Metrohm glass electrode, a Orion 90–05 Ag/AgCl reference electrode and a Wilhelm-type salt bridge containing 0.10 M NMe_4NO_3 solution. A glass-jacketed titration cell (50 mL) completely sealed from the atmosphere was used and the temperature was controlled using a Grant W6 thermostat (25.0 ± 0.1 °C). Atmospheric CO_2 was excluded from the cell during the titration by passing purified N_2 across the top of the experimental solution. The standard base was added through a capillary tip at the surface of the solution by a Metrohm Dosimat 665 burette. The ionic strength of the solutions was kept at 0.10 M with NMe_4NO_3.

2.2.3. Measurements

The $[H^+]$ of the solutions was determined by the measurement of the electromotive force of the cell, as described [25]. The value of K_w was found equal to $10^{-13.80}$ M^2.

Measurements were carried out using 20.00 mL of $\cong 2.50 \times 10^{-3}$ M ligand solutions diluted to a final volume of 30.00 mL, in the absence of metal ions and in the presence of each metal ion for which the C_M:C_L ratios were 1:1 and 1:2. A minimum of two replicate measurements was taken. For tetp solutions lower concentrations were used (about 0.04 mmol), due to its lower water solubility. Each titration curve contained typically 50–60 points in the 2.5–11.5 pH range.

The equilibria involving teta, tritp and tetp (especially the last two ligands) were very slow to be attained, sometimes taking up to a few days. Titrations with automated acquisition were possible in most cases, but for the equilibration in each point of the titration 10–50 min were necessary. The same values of stability constants were obtained either using the direct or the back titration curves. However, the trita/Sm^{3+}, trita/Ho^{3+}, tetp/Sm^{3+} and tetp/Ho^{3+} titrations were also performed by a batch method in pH regions where complexation equilibria were not attained during in-cell titrations. For such purpose, solutions were prepared in separated vials in the 4.0–10.0 pH range and measurements were taken every week till stabilisation of the pH, which occurred generally within 15 days.

2.3. ^{31}P NMR spectroscopy measurements

2.3.1. Reagents and solutions

Ligand solutions were prepared at 0.005–0.010 M. The titrant was a fresh 25% (wt.) solution of NMe_4OH obtained from Aldrich and used pure or diluted to ≅1 M after standardisation by titration with 1.0 M HNO_3.

2.3.2. Equipment and work conditions

The titrations were carried out in a closed titration cell and the titrant was added with a Crison microBU 2031 automatic burette. An Orion 720A measuring instrument fitted with a Metrohm combined glass electrode with Ag^+/AgCl reference electrode was used. Atmospheric CO_2 was excluded from the cell during the titration by passing purified nitrogen across the top of the experimental solution. The ionic strength of the solutions was kept at 0.50 M with NMe_4NO_3. ^{31}P NMR spectra of titration samples were recorded in a Bruker AMX-300 spectrometer at 121.5 MHz and 25.0 ± 0.1 °C.

2.3.3. Measurements

The spectroscopic equilibrium measurements were carried out using 10.00 mL of the ligand solutions. Following each addition of titrant, the pH was measured and a sample of solution was placed in a 5 mm NMR tube adapted with an internal capillary tube containing D_2O for locking and H_3PO_4 for reference purposes. After recording the ^{31}P NMR spectra, the sample volume was returned to the titration cell.

2.3.4. Calculation of equilibrium constants

Overall protonation constants β_i^H were calculated by fitting the potentiometric data obtained for the free ligand to the HYPERQUAD program [32] or the spectroscopic data obtained for the free ligand to the HYPNMR program [33]. Stability constants of the various species formed in solution were obtained from the experimental data corresponding to the titration of solutions of ligands and different metal ions (in different metal:ligand ratios), also using the HYPERQUAD program. To achieve the final model for each system (metal and ligand) all the titrations (at M:L different ratios) and individual points obtained in the out-of-cell experiments were considered together. The initial computations were obtained in the form of overall stability constants, $\beta_{M_mH_hL_l}$ values, $\beta_{M_mH_hL_l} = [M_mH_hL_l] / \{[M]^m[H]^h[L]^l\}$. Mononuclear species ML, MH_iL (i = 1–4) and $MH_{-1}L$ were found for most of the metal complexes of the four macrocyclic compounds (being $\beta_{MH_{-1}L} = \beta_{ML(OH)} \times K_w$). Differences, in log units, between the values of protonated or hydrolysed and non-protonated constants provide the stepwise reaction constants. The errors quoted are the standard deviations of the overall stability constants given directly by the program for the input data, which include all the experimental points of all titration curves.

The two first protonation constants of dotp, tritp and tetp were determined by ^{31}P NMR spectroscopy at ionic strength 0.50 M in NMe_4NO_3, which were the necessary conditions to keep the ionic strength. The values were extrapolated to ionic strength 0.10 M using the Davies equation.

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

^{153}Sm and ^{166}Ho 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 [25]. The specific activity of the radionuclides, after 3 h irradiation and at EOB, were 3–4 mCi/mg for ^{153}Sm and 6–7 mCi/mg for ^{166}Ho.

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

Labelling experimental conditions, such as metal-to-ligand molar ratio, pH, time of incubation and temperature were optimized to achieve high chelation efficiency.

The ^{153}Sm and ^{166}Ho complexes were prepared by dissolving the ligands (5 mg) in 0.4 mL double-distilled water followed by the addition of an adequate amount of ^{153}Sm or ^{166}Ho solutions to achieve a 1:2 metal-to-ligand molar ratio. The pH was adjusted with a freshly prepared 1.0 M NaOH solution. Final ligand concentrations were 24 mM for dota and teta, 18 mM for dotp, tritp and tetp and 20 mM for trita.

Labelling efficiency, reaction kinetics and stability of the radiolanthanide complexes were accomplished by ascending silica gel ITLC strips developed with the mobile phase: MeOH:H_2O:NH_3 (4:4:0.2). 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 also remain at the origin. Thus, identification of colloidal radioactive forms was assessed by ascending thin layer chromatography using silica gel ITLC strips developed with saline. In this system, the radiolanthanide complexes and $^{153}Sm(NO_3)_3$ and $^{166}Ho(NO_3)_3$ migrate with $R_f = 1.0$.

2.6. In vitro studies

2.6.1. In vitro stability

The in vitro stability of the radiolanthanide complexes under physiological conditions was studied at 37 °C in order to detect any radiochemical impurities or free radioactive metal. Thus, the radiochemical purity was evaluated in different physiological media at various time points (up to 5 days). Typically, 50 μL of each ^{153}Sm- or ^{166}Ho complexes were added to 100 μL of different solutions namely: saline, 0.1 M phosphate buffer (pH 7.4), 0.1 M Tris–HCl (tris(hydroxymethyl)aminomethane hydrochloride) buffer (pH 7.4), 0.1 M glycine–HCl (pH 4.0) and human serum. Daily, an aliquot of each mixture was removed and evaluated by ITLC analysis, as described above. The percentage of radiochemical impurities was then calculated.

2.7. Adsorption studies

Adsorption of the ^{153}Sm and ^{166}Ho–tritp complexes onto hydroxyapatite (HA) was accomplished following an adaptation of previously described methods [34,35]. Briefly, 50 μL of each complex (~80 μCi/50 μL) was incubated for 1 h at room temperature with 5, 10, 25, 50 or 75 mg of solid HA and 2 mL of 0.1 M tris buffer (pH 7.4). The liquid and solid phases were separated using a 0.45 μm membrane filter (Millex-HV, Millipore) which was then washed with 8 mL of 0.1 M tris buffer (pH 7.4) (liquid phase). HNO_3 (8 mL of 2% (v/v)) was used to wash the filter and determine the adsorbed fraction retained on the solid HA. The activity in the liquid and solid phases was determined using the ionization chamber.

2.8. Complex charge, lipophilicity and protein binding

The overall charge of the complexes, in 0.1 M tris buffer (pH 7.4), was determined by electrophoresis as previously described [25].

The lipophilicity (log P values) and protein binding were assessed according to the previously described methods [25].

2.9. In vivo studies

2.9.1. Biodistribution studies

Biodistribution studies of the radiocomplexes were performed in female CD-1 mice (randomly bred Charles River, from CRIFFA, Spain) weighing approximately 20–22 g. Animals were injected through tail vein with 100 μL (10–15 MBq/100 μL) of each radiolanthanide complex solution and were sacrificed by cervical dislocation at 30 min, 2 and 24 h post-injection according to a previously described method [25]. 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.).

3. Results and discussion

3.1. Stability constants

The protonation constants of trita and teta (Table 1) and of tritp and tetp (Table 2) were determined at 25 °C and $I = 0.10$ M in NMe_4NO_3. The two first protonation constants of dotp, tritp and tetp were determined by ^{31}P NMR spectroscopy titrations, because these values are very high and could not be determined by direct potentiometry (see Fig. 2 for the corresponding titrations, δ (ppm) versus pH).

The stability constants of the complexes of those ligands with La^{3+}, Sm^{3+} and Ho^{3+} were also determined in the same experimental conditions, and the corresponding values are also collected in Tables 1 and 2. The literature values for dota and dotp with the same metal ions are also listed for comparison reasons, however recommended values for these two ligands do not exist [36]. Indeed, the very high values of stability constants of the metal complexes with dota and dotp together with their slow formation kinetics make the determination very difficult.

The overall basicity of the ligands containing methylphosphonate arms is very high compared with that of the acetate derivatives. This is explained by electrostatic effects and hydrogen bonding formation [25,40]. Additionally the 12-membered macrocyclic derivatives, dota and dotp, present higher overall basicity values than the corresponding 13- and 14-membered macrocycles, which can be explained by differences in hydrogen bonding formation – $^{+}NH\cdots N$ inside the macrocyclic cavity [30,37,40]. The dif-

Table 1
Protonation constants (log β_{H_iL} and log K_{H_iL}[a]) of dota, trita and teta, and stability constants (log β_{MH_iL} and log K_{MH_iL}[a]) of their complexes with lanthanide metal ions

Ion	Species MHL	Dota	Trita		Teta	
		log K_{MH_iL}	log β_{MH_iL}	log K_{MH_iL}	log β_{MH_iL}	log K_{MH_iL}
H^+	011	12.09 [b]	10.97(1)[c]	10.97	10.59(1) [c]	10.59
	021	9.76[b]	20.29(1)[c]	9.32	20.68(1)[c]	10.08
	031	4.56[b]	24.81(2)[c]	4.52	24.80(1)[c]	4.15
	041	4.09[b]	27.81(2)[c]	3.00	28.09(2)[c]	3.29
	051	–	–	–	29.9(1)[c]	1.84
La^{3+}	101	22.9[d]	14.52(9)	14.52	12.15(2)[c]	12.15
	111	–	20.68(3)	6.16	–	–
	121	–	24.62(6)	3.94	24.28(2)	–
	1–11	–	–	–	4.57(4)	−7.58
Sm^{3+}	101	23.0[d]	16.69(9)	16.69	14.15(4)	14.15
	111	–	22.67(2)	5.98	–	–
	121	–	–	–	24.38(4)	–
	1–11	–	8.67(9)	−8.02	6.78(8)	−7.37
	1–21	–	–	–	−0.80(7)	−7.58
Ho^{3+}	101	24.8[d]	17.38(9)	17.38	15.78(3)	15.78
	111	–	23.00(1)	5.62	–	–
	121	–	–	–	24.62(3)	–
	1–11	–	9.30(9)	−8.02	8.75(1)	−7.03
	1–21	–	–	–	1.39(8)	−7.36

$T = 25.0$ °C; $I = 0.10$ M in NMe_4NO_3.
[a] $K_{H_iL} = [H_iL]/([H_{i-1}L] \times [H])$ and $K_{MH_iL} = [MH_iL]/([MH_{i-1}L] \times [H])$; the values in parentheses are standard deviations in the last significant figures.
[b] [29,37].
[c] Determined before in KNO_3 [29,37] and redetermined now in NMe_4NO_3.
[d] Recommended values for these constants does not exist [36], the values shown are only indicative, at r.t. and $I = 1$ M in NaCl [38].

ferent overall basicity of the ligands has direct repercussion in their complexation properties.

All ligands studied form complexes with high, or extremely high, ML thermodynamic stability constants. The complexes of ligands with acetate arms present lower K_{LnL} values than the methylphosphonate derivatives of the corresponding macrocycles. The $pM = -\log[M]$ values, which allow the comparison of the complexometric behaviour of ligands having different overall basicity, confirm also this conclusion [25,40]. Dota and dotp do not fit in this trend, with dota lanthanide complexes exhibiting higher pM values. However, without recommended values of stability constants for the lanthanide complexes of dota and dotp it is useless to establish final conclusions [36].

Additionally, the ligands containing methylphosphonate arms form several protonated complexes with lanthanides. Therefore, the completely deprotonated ML complexes only exist as the main species at pH values $\geqslant 9$, while the ML complexes of the ligands with acetate arms are formed at pH values about 6–7, see the species distribution diagrams for teta/Ho^{3+} and tetp/Ho^{3+} in Fig. 3, or identical diagrams for trita/Sm^{3+} and tritp/Sm^{3+} in [40].

The most interesting point to be stressed from the values of Tables 1 and 2 is the significant decrease of the K_{ML} (and pM) values for the lanthanide complexes with the increase of the cavity size [40]. These values decrease gradually from dota to teta and from dotp to tetp for the same metal ion.

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

^{153}Sm and ^{166}Ho obtained with high radionuclidic purity, as confirmed by the typical γ-ray spectra shown in Fig. 4 (major γ peaks for ^{153}Sm, 40.8, 41.4, 46.9, 48.2, 69.6, 75.4, 97.3 and 103.1 keV and for ^{166}Ho, 55.5, 57.1 and 80.5 keV) [41], were used to prepare complexes with the tetraazamacrocycles.

The reaction conditions were optimized in order to obtain ^{153}Sm and ^{166}Ho complexes with high radiochemical purity. The labelling conditions and chelation efficiencies of the different complexes, expressed as percentage, are summarized in Table 3. The labelling conditions for $^{153}Sm/^{166}Ho$–dota and $^{153}Sm/^{166}Ho$–dotp complexes are also given for comparison.

The kinetics was found to be dependent on the ligand macrocyclic cavity size, nature of pendant arms and concentration, as well as on the pH and temperature of reaction mixture. For all the ligands, at 1:1 metal-to-ligand molar ratio the labelling was not complete, maximum complex formation was only achieved at 1:2 metal-to-ligand molar ratio. The labelling efficiency was studied over a pH range 6–10. For $^{153}Sm/^{166}Ho$–trita and $^{153}Sm/^{166}Ho$–teta maximum complexation (>98%) was achieved at room temperature (r.t.) and at pH 6–7, values at which the main species are ML, according to the species distribution diagrams (Fig. 3). However, with the methylphosphonate derivatives maximum complexation was only achieved at

Table 2
Protonation constants (log β_{H_iL} and log K_{H_iL}[a]) of dotp, tritp and tetp, and stability constants (log β_{MH_iL} and log K_{MH_iL}[a]) of their complexes with lanthanide metal ions

Ion	Species MHL	Dotp		Tritp		Tetp	
		log β_{MH_iL}	log K_{MH_iL}	log β_{MH_iL}	log K_{MH_iL}	log β_{MH_iL}	log K_{MH_iL}
H^+	011	14.65(2)[b]	14.65	13.20(1)[b]	13.20	–	–
	021	27.05(2)[b]	12.40	25.66(1)[b]	12.46	25.28(2)[b]	–
	031	36.33[c]	9.28	34.37(1)	8.71	34.13(2)	8.85
	041	44.42[c]	8.09	41.70(1)	7.33	41.81(2)	7.68
	051	50.54[c]	6.12	47.83(2)	6.13	48.04(2)	6.23
	061	55.76[c]	5.22	52.85(2)	5.02	53.37(2)	5.33
	071	–	–	55.22(2)	2.37	55.65(3)	2.28
La^{3+}	101	27.6 [d]	27.6	21.00(5)	21.00	18.02(9)	18.02
	111	35.3[d]	7.7	29.74(4)	8.74	27.29(9)	9.27
	121	42.0[d]	6.7	37.93(5)	8.19	35.94(9)	8.65
	131	47.6[d]	5.6	45.21(4)	7.28	44.22(7)	8.28
	141	52.3[d]	4.7	50.63(1)	5.42	50.54(3)	6.32
	151	–	–	–	–	53.91(5)	3.37
	1–11	–	–	10.61(5)	−10.39	7.38(9)	−10.64
Sm^{3+}	101	28.1[d]	28.1	23.83(8)	23.83	19.11(9)	19.11
	111	35.7[d]	7.6	32.53(8)	8.70	28.74(7)	9.63
	121	42.0[d]	6.3	40.66(7)	8.13	37.32(6)	8.58
	131	47.4[d]	5.4	47.08(5)	6.42	45.09(5)	7.77
	141	51.8[d]	4.4	51.39(2)	4.31	51.24(3)	6.15
	151	–	–	–	–	54.59(4)	3.35
	1–11	–	–	14.87(8)	−8.96	10.33(8)	−8.78
Ho^{3+}	101	29.2[d]	29.2	24.07(9)	24.07	20.03(9)	20.03
	111	37.5[d]	8.3	33.17(9)	9.10	29.55(9)	9.52
	121	44.4[d]	6.9	41.05(8)	7.88	38.80(9)	9.25
	131	50.0[d]	5.6	47.53(8)	6.48	46.38(8)	7.58
	141	54.5[d]	4.5	52.24(7)	4.71	51.98(7)	5.60
	151	–	–	–	–	55.08(9)	3.10
	1–11	–	–	14.67(9)	−9.40	9.94(9)	−10.09

$T = 25.0$ °C; $I = 0.10$ M in NMe_4NO_3.
[a] $K_{H_iL} = [H_iL]/([H_{i-1}L][H])$ and $K_{MH_iL} = [MH_iL]/([MH_{i-1}L][H])$; the values in parentheses are standard deviations in the last significant figures.
[b] Determined from ^{31}P NMR spectroscopic titration data.
[c] Determined before [30], and redetermined in this work.
[d] [39].

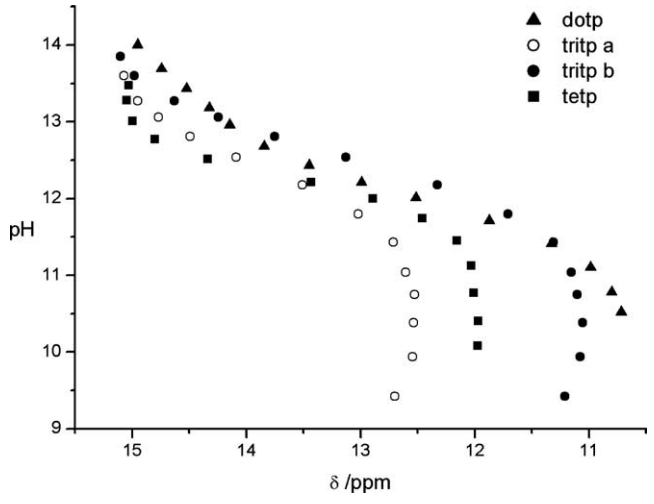

Fig. 2. ^{31}P NMR spectroscopy resonance shifts of the phosphonate groups of dotp, tritp and tetp as a function of pH. For tritp two resonances, a and b, were observed.

60–70 °C and at pH values of 9 for $^{153}Sm/^{166}Ho$–tritp (>98%), 7 for ^{153}Sm–tetp (>80%) and 8 for ^{166}Ho–tetp (>80%) (Table 3). As can be seen in the species distribution diagrams, at these pH values the deprotonated ML complex is the main species for $^{153}Sm/^{166}Ho$–tritp, while in the case of ^{153}Sm–tetp and ^{166}Ho–tetp at pH 7 and 8 the main species present are MLH_3 and MLH_2, respectively. The relatively low yield obtained for $^{153}Sm/^{166}Ho$–tetp at these pH values, led us to explore the possibility of using pH >9 but, in both cases, we observed the formation of unsoluble species without improving the radiochemical yield. Using the experimental conditions indicated in Table 3 and based on ITLC analysis, we found that the radiochemical impurity present in the reactions with tetp is not free lanthanide but some colloidal unidentified species. Several attempts have been made to separate $^{153}Sm/^{166}Ho$–tetp from the reaction mixture, using anion exchange chromatography (Sephadex C 25), gel filtration (Sephadex G 25) and/or solid phase extraction (Sep-pak C18 cartridges, Millipore). However, we never succeeded and most of the times the free ligand was recovered, as shown by ^1H and

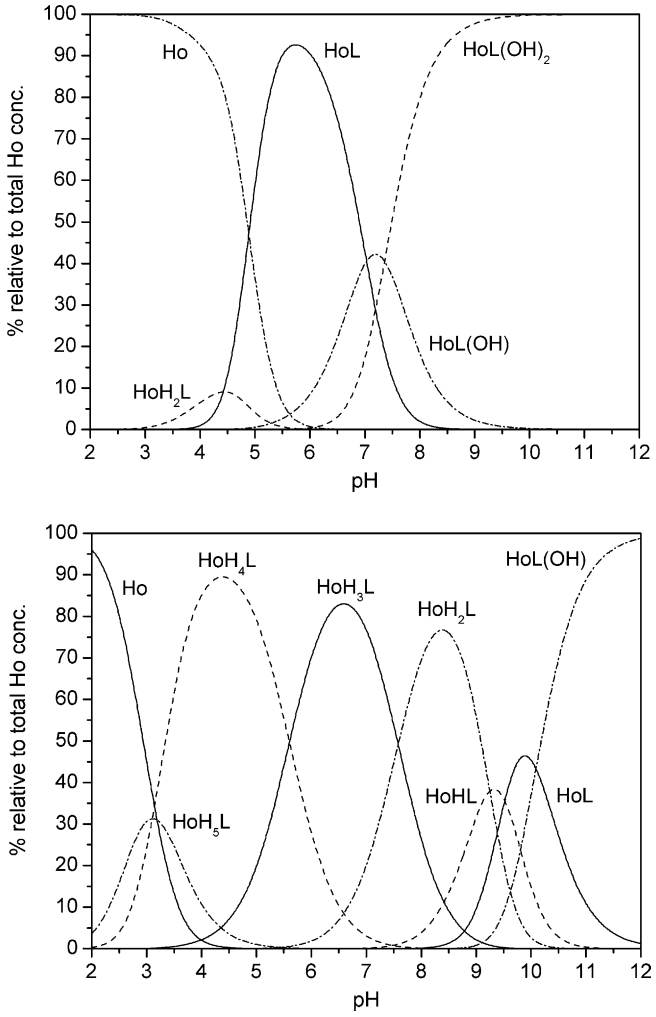

Fig. 3. Species distribution diagrams calculated for 1:2 (M:L) ratio for the complexes of Ho^{3+} with L = teta (top) and L = tetp (bottom). $C_M = 3.0 \times 10^{-4}$ M.

^{13}C NMR spectroscopy. These results compare with previous studies described by Das et al. [42]. These authors also tried to prepare ^{177}Lu–tetp, at pH 9, but the maximum yield achieved was only 75%.

3.3. In vitro stability, charge, lipophilicity and protein binding

The stability of all the radiolanthanide complexes was evaluated for a period of 5 days at 37 °C in the presence of saline, phosphate buffer (pH 7.4), 0.1 M tris buffer (pH 7.4), 0.1 M glycine–HCl solution (pH 4.0) and human serum. These studies indicate that $^{153}Sm/^{166}Ho$ complexes of the ligands with acetate arms were stable up to five days as no significant release of free metal or appearance of radioactive colloidal species could be detected. Only for $^{153}Sm/^{166}Ho$–teta complexes some instability was found in phosphate buffer. The $^{153}Sm/^{166}Ho$–tritp complexes are stable up to five days in the presence of all physiological media, except in saline.

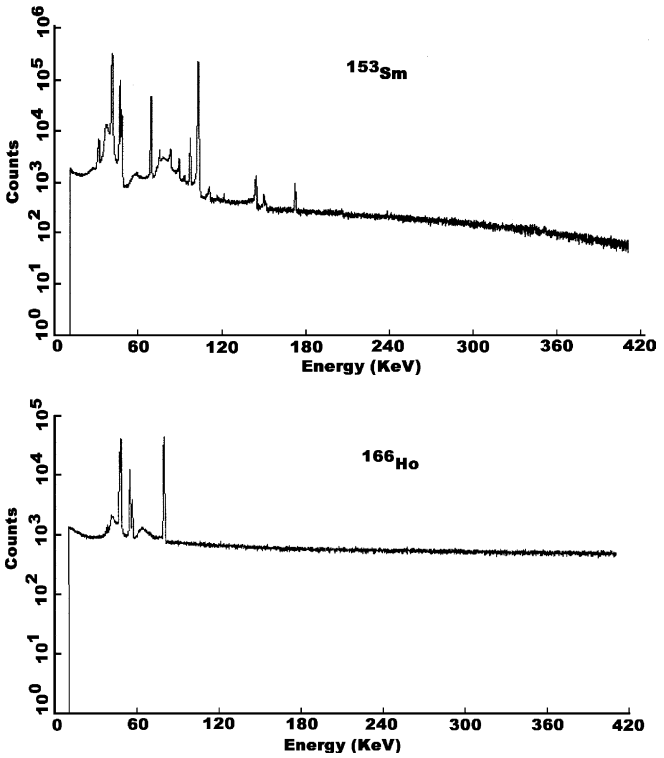

Fig. 4. Typical γ-ray spectrum of ^{153}Sm and ^{166}Ho produced at the Portuguese Research Reactor (RPI).

By electrophoresis, in Tris–HCl buffer (pH 7.4, 0.1 M), the overall charge of all the $^{153}Sm/^{166}Ho$ complexes was found to be negative.

The lipo-hydrophilic character of the $^{153}Sm/^{166}Ho$ radiolanthanide complexes was evaluated based on the octanol/saline partition coefficients ($\log P$ values) [25]. As can be seen in Table 4, all the radiolanthanide complexes present high hydrophilic character ($\log P < -1$), certainly due

Table 3
Labelling conditions for ^{153}Sm– and ^{166}Ho–labelled tetraazamacrocyclic complexes

Ligand	^{153}Sm–macrocyclic complexes		^{166}Ho–macrocyclic complexes	
	Labelling conditions	Labelling efficiency	Labelling conditions	Labelling efficiency
Dota	5 min r.t., pH 6/7	>98%	5 min r.t., pH 7	>98%
Trita	5 min r.t., pH 6/7	>98%	5 min r.t., pH 6/7	>98%
Teta	2.5 h r.t., pH 6	>98%	2.5 h r.t., pH 6/7	>98%
Dotp	1 h 60–70 °C, pH 8	>98%	1 h 60–70 °C, pH 8	>98%
Tritp	2 h 60–70 °C, pH 9	>98%	2 h 60–70 °C, pH 9	>98%
Tetp	2.5 h 60–70 °C, pH 7	>80%	2.5 h 60–70 °C, pH 8	>80%

r.t.: room temperature.

to the high degree of ionisation of the acetate and phosphonate groups.

In order to get a better understanding of the biokinetics of our ^{153}Sm/^{166}Ho complexes their binding to human serum proteins was evaluated by gel filtration. The results obtained indicated a low binding to human serum proteins for all the radiocomplexes: 1.4–3.8% for ^{166}Ho– and 7–14% for ^{153}Sm–radiocomplexes, respectively (Table 4).

3.4. Adsorption studies

The degree of exchange of ^{153}Sm/^{166}Ho complexes with HA was studied for the ligands with methylphosphonate arms, once the complexes of the acetate derivative ligands themselves have very little affinity for the bone matrix [43]. As can be seen in Fig. 5, the adsorption curves of ^{153}Sm/^{166}Ho–tritp complexes onto HA are comparable, indicating that a maximum binding of ~80% is reached when 10 mg of HA is used. These data are encouraging for in vivo studies, and compare well with the values found for the previously described ^{153}Sm–dotp complexes [11,35].

3.5. Biodistribution studies

The biodistribution of ^{153}Sm/^{166}Ho complexes was assessed in CD-1 mice at 30 min and 2 h upon administration, and upon 24 h for the complexes of ligands with the phosphonate arms. Tissue distribution data of the radiolanthanide complexes with the acetate derivative ligands was expressed as percentage of injected dose per gram of organ. The uptake and clearance from most relevant organs are shown in Fig. 6. The ^{153}Sm/^{166}Ho–trita complexes present a similar pattern, showing a rapid clearance from most organs including blood and muscle. For both a very high rate of total radioactivity excretion from whole animal body was found (>80% and 90% at 30 min and 2 h after administration, respectively). The relative high kidney uptake (1.3% of D.I. at 2 h p.i.) associated to the high total radioactivity excretion indicated that those complexes clear, almost exclusively, through kidney pathway. ^{166}Ho–teta complex presents also a similar biological distribution with a rapid total radioactivity excretion (>80% at 2 h after administration). A quite different behaviour was observed for the ^{153}Sm–teta complex. This compound

Table 4
Human serum protein binding and lipo-hydrophilic character (log P) of ^{153}Sm– and ^{166}Ho–labelled tetraazamacrocyclic complexes

Ligand	^{153}Sm-macrocyclic complexes		^{166}Ho-macrocyclic complexes	
	% Protein binding	log *P*	% Protein binding	log *P*
Dota	7.0	−2.02	1.4	−1.64
Trita	7.0	−1.93	2.6	−1.53
Teta	7.8	−1.75	3.8	−1.45
Dotp	–	−2.00	–	−1.90
Tritp	14	−1.48	2	−1.08

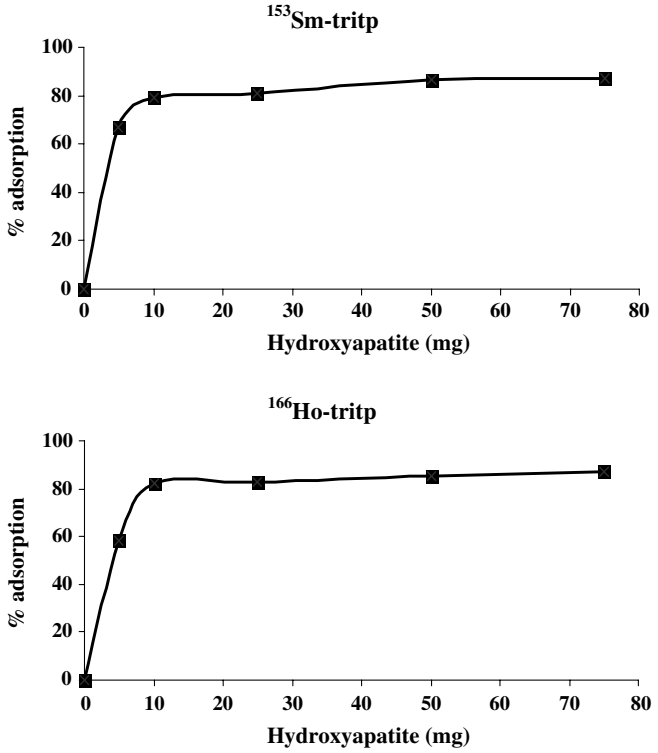

Fig. 5. Adsorption of ^{153}Sm/^{166}Ho–tritp complexes as a function of the amount of hydroxyapatite (HA).

presents a slow rate of total radioactivity excretion (approximately 30% and 40% at 30 min and 2 h after administration, respectively) associated to a slow clearance from blood stream and muscle. A significant liver uptake that increases over time was also found, indicating eventual in vivo formation of some radiochemical species of colloidal/polymeric nature.

Due to the importance of dota as a bifunctional chelator, namely for ^{90}Y, ^{111}In and ^{177}Lu, we decided to study the biological behaviour of ^{153}Sm/^{166}Ho–dota complexes in the same animal model. We found that trita and dota complexes have a quite similar biodistribution profile, with no significant differences in the rates of organs clearance and whole body excretion. However, the ^{166}Ho–teta complex presents a rate of radioactivity elimination slightly slower than observed for trita and dota complexes (Fig. 6).

To evaluate the in vivo stability of the lanthanide complexes, blood and urine samples were taken at sacrifice time and were analysed by ITLC. ^{153}Sm/^{166}Ho–trita and ^{166}Ho–teta complexes are stable in blood and are excreted as an intact complex, similar to what has been found for ^{153}Sm/^{166}Ho–dota. However, the ^{153}Sm–teta complex is not stable in vivo and radiochemical impurities other than the intact complex could be detected in blood and urine. These impurities, which are not free metal, may be responsible for the in vivo hepatic retention. In fact our chemical studies revealed that the pSm value for teta is lower than for trita (8.71 for teta and 10.71 for trita), while the pHo for both ligands are similar (11.20 for trita and 10.67 for

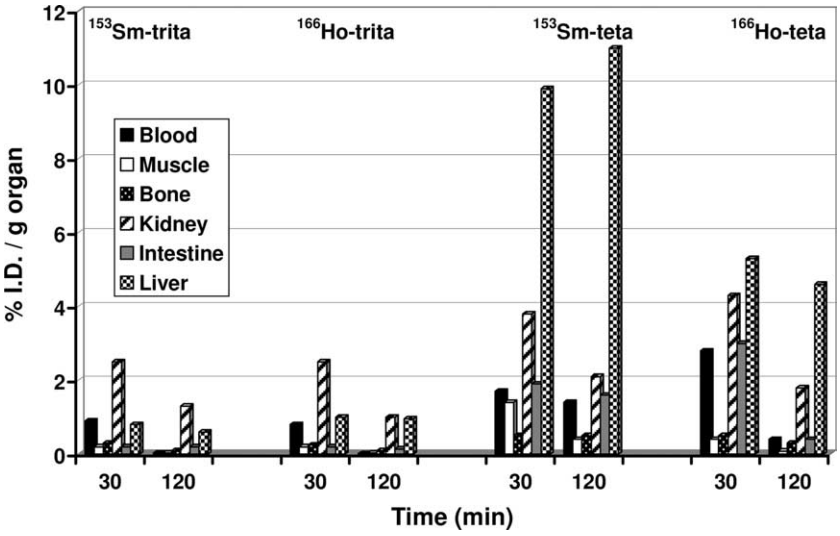

Fig. 6. Biodistribution data, expressed as percent of injected dose per gram of organ (% I.D. ± SD) of ^{153}Sm/^{166}Ho complexes with 13- and 14-membered macrocyclic ligands containing methylcarboxylate pendant arms, 30 min and 2 h after intravenous (i.v.) administration in female CD-1 mice ($n = 3$–4).

teta), with the pM values being calculated for the complexes at pH = 7.4 and for 100% excess of free ligand, $C_L = 2C_M = 2.0 \times 10^{-5}$ M [40].

Biodistribution data for ^{166}Ho–tritp and a comparison between the uptake of ^{153}Sm– and ^{166}Ho–tritp complexes in most significant organs are presented in Fig. 7 and Table 5, respectively. The ^{166}Ho–tritp complex is rapidly taken by the main excretory organs, kidney and hepatobiliar tract, being rapidly eliminated. Therefore, a rapid total excretion and washout from all the other organs including blood was observed. A pronounced bone uptake at 30 min (ca 6.5%) which slightly decreases over time, remains still significant after 24 h (ca 5.5%). Due to this biodistribution profile a quite favourable bone/blood and bone/muscle ratios were achieved (Table 5). Bone uptake was also observed after ^{153}Sm–tritp complex

Table 5
Biodistribution data of ^{153}Sm– and ^{166}Ho–tritp complexes, expressed as percent of injected dose per gram organ (% I.D. ± SD) for the most significant organs, 2 and 24 h after intravenous (i.v.) administration in female CD-1 mice ($n = 3$–4)

Organ	^{153}Sm–tritp		^{166}Ho–tritp	
	2 h	24 h	2 h	24 h
Blood	5.8 ± 0.7	0.4 ± 0.1	0.29 ± 0.09	0.02 ± 0.01
Liver	10.0 ±0.9	11.0 ± 1.9	0.23 ± 0.03	0.26 ± 0.05
Spleen	33.0 ± 3.1	3.2 ± 0.9	0.33 ± 0.21	0.32 ± 0.09
Muscle	0.7 ± 0.2	0.4 ± 0.2	0.09 ± 0.01	0.07 ± 0.02
Bone	2.2 ± 0.2	1.6 ± 0.5	5.5 ± 0.8	5.5 ± 1.0
Bone/ blood	0.4	3.9	22.7	274
Bone/ muscle	3.1	3.8	54.8	84
Excretion (% I.D.)	18.0 ± 4.4	52.3 ± 3.1	77.8 ± 1.0	78.0 ± 1.6

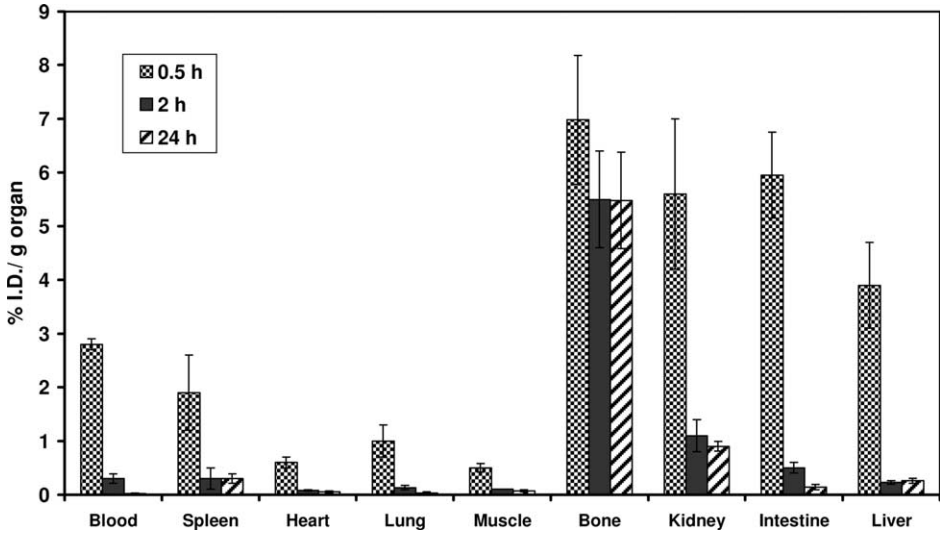

Fig. 7. Biodistribution data, expressed as percent of injected dose per gram organ (% I.D. ± SD) of ^{166}Ho–tritp complex, 30 min, 2 and 24 h after intravenous administration in female CD-1 mice ($n = 3$–4).

administration, although with a faster decrease of radioactivity, and the rate of total excretion and clearance from main organs, like blood and muscle, was significantly slower, leading to a less favourable bone/non-target ratios (Table 5). High hepatic and splenic uptake also suggest in vivo formation and retention of radiochemical impurities of colloidal nature. In order to get a better insight on the in vivo remarkable differences found for these complexes, we carried out some urine and blood ITLC analysis. Data from these studies revealed that the ^{166}Ho–tritp complex was stable in blood and it was almost all excreted as an intact complex. In contrast, some impurities were found in the blood analysis of mice after ^{153}Sm–tritp complex administration. This observed instability in blood can be explained by the possible binding of the complex to carrier proteins, already reported for other radiolanthanide complexes [25].

As referred above, the ^{153}Sm/^{166}Ho–dotp complexes have been previously described as promising bone agents [9,11]. In order to get comparable results, ^{153}Sm/^{166}Ho–dotp complexes were synthesized and their biological behaviour was analysed in the animal model used for ^{166}Ho–tritp complex. Fig. 8 presents a summary of the bone uptake found for these three complexes.

^{153}Sm/^{166}Ho–dotp complexes showed bone uptake with high rate of total excretion (ca 80%), rapid blood clearance and minimal uptake in all of the major organs, leading to high bone/blood and bone/muscle rates in agreement with the published results obtained in different animals [11]. As can be seen in Fig. 8, the ^{166}Ho–tritp complex exhibits a bone uptake comparable to what has been found for ^{166}Ho–dotp complex, being the values higher than those found for ^{153}Sm–dotp complex. The almost constant bone uptake values, between 2 and 24 h post-injection (p.i.), indicate that the ^{166}Ho–tritp complex can be promising as a bone pain palliation agent.

3.6. Concluding remarks

All the ligands studied in this work (trita, teta, tritp and tetp) form lanthanide complexes with high or extremely high ML thermodynamic stability constants. For the same lanthanide ion the K_{LnL} values decrease with the increase of the cavity size of the macrocycle, and those values are lower for trita and teta than for the corresponding tritp and tetp complexes. The pM values, which take into account the overall basicity of the ligands, confirmed these trends [40].

Using a 1:2 metal-to-ligand molar ratio all the macrocycles form complexes with ^{153}Sm and ^{166}Ho ions, but the kinetics of the complex formation with ligands having methylphosphonate arms is slower than for those with methylcarboxylate substituents. For the same metal ion the kinetics also decreases with the increase of the macrocyclic cavity size. For the ligands with carboxylate arms the maximum yield (>98%) was found at pH 6–7, indicating that the main species formed are the deprotonated ML complexes. A similar behaviour was found for ^{153}Sm/^{166}Ho–tritp complexes but at pH = 9. However, for ^{153}Sm/^{166}Ho–tetp complexes precipitation occurred at high pH values, the maximum yields (80%) being achieved at pH 7 and 8, indicating that the main species formed are MLH_3 and MLH_2. All the radiolanthanide complexes isolated in high yield (>98%) are hydrophilic, present an overall negative charge and a low protein binding. In vivo, the complexes present a high whole body radioactivity excretion, and a promising biological profile, being the ^{153}Sm–teta the less promising in terms of rate of total excretion, clearance from blood and muscle and also in terms of in vivo stability. The differences observed in the tissue distribution of the complexes with methylcarboxylates and methylphosphonates are mainly related with the degree of bone uptake. The ^{153}Sm/^{166}Ho–tritp complexes have considerable bone uptake, and the values found for

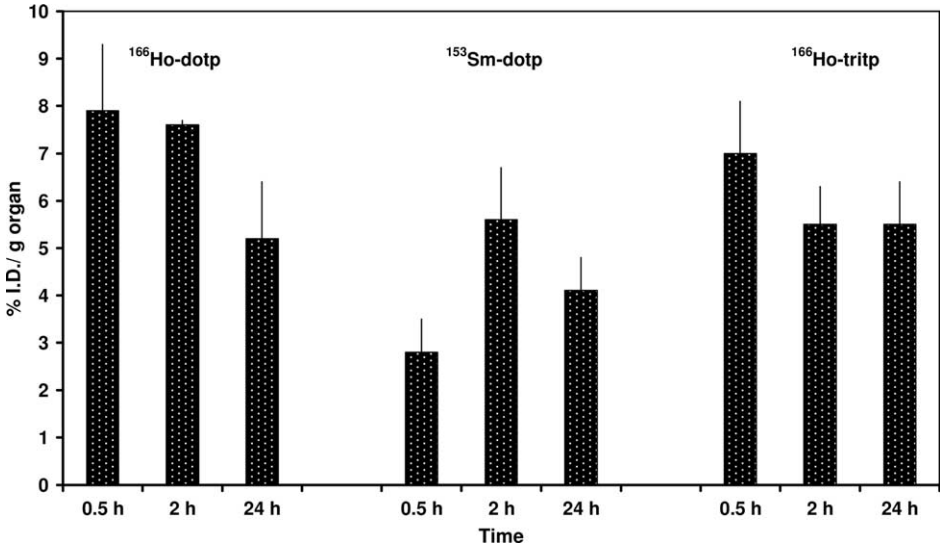

Fig. 8. Bone uptake, expressed as percent of injected dose per gram organ (% I.D. ± SD) of radiolanthanide complexes with macrocyclic ligands containing methylphosphonate pendant arms, 30 min, 2 and 24 h after intravenous (i.v.) administration in female CD-1 mice ($n = 3$–4).

^{166}Ho–tritp are comparable to the values found for ^{166}Ho–dotp which is under clinical studies. The biological behaviour of ^{153}Sm/^{166}Ho–trita make these complexes promising as potential therapeutic agents when linked to a carrier biomolecule to target selectively the diseased site.

Acknowledgements

The authors acknowledge the financial support from Fundação para a Ciência e a Tecnologia (FCT) and POCTI, with co-participation of the European community fund FEDER (project no. POCTI/2000/CBO/35859). COST ACTION D18 is also acknowledged. The authors thank the ITN Portuguese Research Reactor Group for the production of ^{153}Sm and ^{166}Ho and Dr. A. Gouveia by γ-spectrometry analysis.

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