J Radioanal Nucl Chem (2013) 295:979–986
DOI 10.1007/s10967-012-2034-1

Development and evaluation of a ^{166}holmium labelled porphyrin complex as a possible therapeutic agent

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Received: 13 May 2012 / Published online: 8 August 2012

Abstract Porphyrins are interesting derivatives with low toxicity, tumor avidity and rapid wash-out suggested as potential radiopharmaceuticals in radiolabeled form. In this work, [^{166}Ho] labeled 5,10,15,20-tetrakis(phenyl) porphyrin ([^{166}Ho]-TPP) was prepared using [^{166}Ho]$HoCl_3$ and 5,10,15,20-tetrakis(phenyl)porphyrin (H_2TPP) for 12 h at 50 °C (radiochemical purity: >95 ± 2 % ITLC, >99 ± 0.5 % HPLC, specific activity: 0.9–1.1 GBq/mmol). Stability of the complex was checked in final formulation and human serum for 48 h. The partition coefficient was calculated for the compound (log $P = 2.01$). The biodistribution of the labeled compound in vital organs of wild-type rats was studied using scarification studies and SPECT. A detailed comparative pharmacokinetic study performed for ^{166}Ho cation and [^{166}Ho]-TPP performed up to 24 h. The complex is mostly washed out from the circulation through kidneys and in less extends from the liver. The kidney:blood, kidney:liver and kidney:muscle ratios 4 h post injection were 14, 3.6 and 7.38 respectively.

Keywords Porphyrins · ^{166}Ho · Half life · Biodistribution · SPECT

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Introduction

Various metal-porphyrin complexes have shown interesting tumor-avid activity in vitro and in vivo and have found their ways into clinical studies, for instance Motexafin gadolinium, a radiation sensitizer, is used in the therapy of brain tumors [1], also a boronated porphyrin is used in boron-neutron capture therapy [2]. Also tumor accumulation of some indium–porphyrin complexes has already been reported [3]. Radiolabeled therapeutic porphyrins have been developed for the therapeutic purposes such as, ^{109}Pd-protoporphyrins [4], ^{109}Pd-porphyrins [5], ^{109}Pd-derivitized porphyrins [6] and ^{188}Re-porphyrins [7], however no significant therapeutic effects were reported.

Diagnostic radiolabeled porphyrins have also been reported including ^{99m}Tc-porphyrin conjugates [8, 9], ^{111}In-porphine [10] and ^{166}Ho–porphyrin complex [11], however while all demonstrate high hepatotoxicity.

Despite, the general interest in application of certain tumor imaging agents such as ^{18}FDG, many limitations exist. These include healing processes, inflammatory diseases such as arthritis or Crohn's disease [12], infectious and granulomatous diseases such as tuberculosis or sarcoidosis [13]. The studies also show that all types of WBC, as well as granulation tissue may show highly increased FDG uptake. On the other hand, the use of FDG-PET in the diagnosis of localized prostate cancer has proven to be disappointing primarily because most prostate cancers have a relatively low glycolytic rate and therefore do not accumulate high concentrations of the radiotracer FDG. In addition, excreted FDG activity accumulating in the ureters and bladder may limit the evaluation of adjacent structures such as the prostate and pelvic lymph nodes.

The high expenses and international limitations in establishing PET cameras in 86 % of developing and

under-developed countries, suggest continuation of kit-based radiopharmaceuticals application used in SPECT.

Many beta-emitters such as ^{153}Sm, ^{177}Lu and ^{166}Ho can be produced in reasonable amounts using (n, gamma) reactions. ^{166}Holmium (E_{β^-} max = 1.84 MeV, $T_{1/2}$ = 26.8 h) is one of the most interesting radionuclides for targeted therapy modalities.

^{166}Ho-radiopharmaceuticals have been developed and used in the therapy of various diseases and malignancies. Holmium-166 microspheres are widely used for the treatment of liver malignancies [14]. The effectiveness of ^{166}Ho–chitosan complex therapy for malignancies such as gliomas [15] and prostate cancer [16] has been successfully approved. This radionuclide has also been extensively used in radiation synovectomy in form of ^{166}Ho-macroaggregates [17] to destroy the inflamed synovium. ^{166}Ho DOTMP is a tetraphosphonate compound localizing rapidly to bone surface for bone palliation therapy [18].

Endovascular beta irradiation therapy is a therapeutic module using ^{166}Ho by an angio-catheter [19] applying various Ho-complexes such as ^{166}Ho-DTPA in the stenosis site [20]. Intra-arterial hepatic infusion of ^{166}Ho-oxine-lipiodol was used to deliver localized doses of ionizing radiation to liver cancer cells after it is selectively retained in the vascular periphery of the proliferating cells [21].

^{166}Ho patches have been used in the treatment of Bowen's disease when applied to the surface of skin cancers [22].

Due to the interesting pharmacological properties of porphyrins such as solubility in serum, rapid wash-out, tumor avidity and feasible complexation with various bi/tri-valent metals [23], the idea of developing a possible tumor targeting agent by incorporating ^{166}Ho into a suitable porphyrin ligand, i.e. H_2TPP was investigated (Fig. 1).

In this work we report, synthesis, radiolabeling, quality control, stability, partition coefficient determination and biodistribution studies (using SPECT and scarification) of ^{166}Ho-TPP in wild-type rats. The time/activity diagrams for the labeled compound in vital organs have been plotted compared to holmium cation.

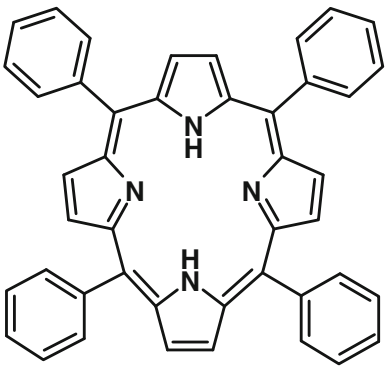

Fig. 1 Structure of H_2TPP

Experimental

Production of ^{166}Ho was performed at the Tehran Research Reactor (TRR) using ^{165}Ho(n, gamma)^{166}Ho nuclear reaction. Natural holmium nitrate with purity of >99.99 % was obtained from ISOTEC Inc.. All chemicals were purchased from Sigma-Aldrich Chemical Co. U.K.. Radiochromatography was performed by Whatman paper using a thin layer chromatography scanner, Bioscan AR2000, Paris, France. Analytical HPLC to determine the specific activity was performed by a Shimadzu LC-10AT, armed with two detector systems, flow scintillation analyzer (Packard-150 TR) and UV–Visible (Shimadzu) using Whatman Partisphere C-18 column 250 × 4.6 mm (Whatman Co. NJ, USA). Calculations were based on the 80.6 keV peak of ^{166}Ho. All values were expressed as mean ± standard deviation (Mean ± SD) and the data were compared using Student t test. Animal studies were performed in accordance with the United Kingdom Biological Council's Guidelines on the Use of Living Animals in Scientific Investigations, second edition.

Production and quality control of $^{166}HoCl_3$ solution

Holmium-166 was produced by neutron irradiation of 100 μg of natural $^{165}Ho(NO_3)_3$ (^{165}Ho, 99.99 % from ISOTEC Inc.) according to reported procedures [24] in the Tehran Research Reactor at a thermal neutron flux of 1×10^{13} n.cm^{-2} s^{-1}. Specific activity of the produced ^{166}Ho was 5 GBq/mg after 20 h of irradiation. The irradiated target was dissolved in 200 μl of 1.0 M HCl, to prepare $^{166}HoCl_3$ and diluted to the appropriate volume with ultra pure water, to produce a stock solution. The mixture was filtered through a 0.22 μm biological filter and sent for use in the radiolabeling step. The radionuclidic purity of the solution was tested for the presence of other radionuclides using beta spectroscopy as well as HPGe spectroscopy for the detection of various interfering beta and gamma emitting radionuclides. The radiochemical purity of the $^{166}HoCl_3$ was checked using two solvent systems for ITLC (A: 10 mM DTPA pH 4 and B: ammonium acetate 10 %:methanol (1:1)). Gamma spectroscopy of the final sample was carried out counting in an HPGe detector coupled to a Canberra™ multi-channel analyzer for 1,000 s.

Preparation of tetraphenyl porphyrin ($TPPH_2$)

This compound was prepared according to the reported method using freshly distilled benzaldehyde, pyrrole and propionic acid followed by oxidation [25]. Yield; 20 %, m.p. >248–250 °C. ^{1}H NMR ($CDCl_3$) δ (ppm) −2.8 (2 H, NH), 7.71–7.82 (12 H), 8.14–8.27 (8 H), 8.85 (8 H). ^{13}C-NMR ($CDCl_3$) δ (ppm) 120.20 (C), 126.74 (CH), 127.76

(CH), 131.16 (CH), 134.62 (CH), 142.22 (C), 145.6 (C).UV (toluene) λ_{max} (ε) = 418 nm (413200), 514 (19060), 549 (8080), 594 (5380), 648 (3870). IR (KBr) 3320, 3055, 3025, 1595.

Preparation of [^{166}Ho]-TPP

The acidic solution (2 ml) of [^{166}Ho]$HoCl_3$ (111–333 MBq, 3–9 mCi) was transferred to a 3 ml-borosilicate vial and heated to dryness using a flow of N_2 gas at 50–60 °C. Fifty microlitres of TPP in absolute ethanol (5 mg/ml $\approx$320 nmol) was added to the holmium-containing vial followed by the addition of acetate buffer pH 5.5 (450 µl). The mixture refluxed at 60 °C for 12 h. The active solution was checked for radiochemical purity by ITLC and HPLC. The final solution was then passed through a 0.22 µm filter and pH was adjusted to 5.5–7.

Quality control of [^{166}Ho]-TPP

Radio thin layer chromatography

A 5 µl sample of the final fraction was spotted on a chromatography Whatman No. 2 paper, and developed in mobile phase mixture, 10 % NH_4OAc and methanol 1:1.

High performance liquid chromatography

HPLC was performed with a flow rate of 1 ml/min, pressure: 130 kgF/cm^2 for 20 min. HPLC was performed on the final preparation using a mixture of water:acetonitrile 3:2 (v/v) as the eluent by means of reversed phase column Whatman Partisphere C_{18} 4.6 × 250 mm.

Determination of partition coefficient

Partition coefficient (log *P*) of [^{166}Ho]-TPP was calculated followed by the determination of *P* (*P* = the ratio of specific activities of the organic and aqueous phases). A mixture of 1 ml of 1-octanol and 1 ml of isotonic acetate-buffered saline (pH = 7) containing approximately 3.7 MBq of the radiolabeled holmium complex at 37 °C was vortexed 1 min and left 5 min. Following centrifugation at >1,200×*g* for 5 min, the octanol and aqueous phases were sampled and counted in an automatic well-type counter. A 500 µl sample of the octanol phase from this experiment was shaken again two to three times with fresh buffer samples. The reported log *P* values are the average of the second and third extractions from three to four independent measurements.

Stability tests

The stability of the complex was checked according to the conventional ITLC method [26]. A sample of [^{166}Ho]-TPP (37 MBq) was kept at room temperature for 2 days while being checked by ITLC at time intervals in order to check stability in final product using above chromatography system. For serum stability studies, to 36.1 MBq (976 µCi) of [^{166}Ho]-TPP was added 500 µl of freshly collected human serum and the resulting mixture was incubated at 37 °C for 48 h, aliquots (5-µl) were analyzed by ITLC.

Biodistribution in wild-type rats

The distribution of the radiolabeled complex among tissues was determined for wild-type rats immediately after imaging. The total amount of radioactivity injected into each animal was measured by counting the 1-ml syringe before and after injection in a dose calibrator with fixed geometry.

The animals were sacrificed using the animal care protocols at selected times after injection (2–24 h), the tissues (blood, heart, lung, brain, intestine, feces, skin, stomach, kidneys, liver, muscle and bone) were weighed and rinsed with normal saline and their specific activities were determined with a HPGe detector equipped with a sample holder device as percent of injected dose per g of tissues. Blood samples were rapidly taken from rodent aorta after scarification.

Imaging of [^{166}Ho]-TPP in wild-type rats

Images were taken 2, 8 and 24 h after administration of the radiopharmaceutical by a dual-head SPECT system. The mouse-to-high energy septa distance was 12 cm. Images were taken from both normal and tumor bearing rats. The useful field of view (UFOV) was 540 × 400 mm.

Results and discussion

Production and quality control of ^{166}Ho

The radionuclide was prepared in a research reactor according to regular methods with a range of specific activity 3–5 MBq/mg for radiolabeling use, after counting the samples on an HPGe detector for 5 h and two major photons (5.4 % of 80.68 keV and 0.9 % of 1379.94 keV) were observed. The radioisotope was dissolved in acidic media as a starting sample and was further diluted and evaporated for obtaining the desired pH and volume followed by sterile filtering (Fig. 2).

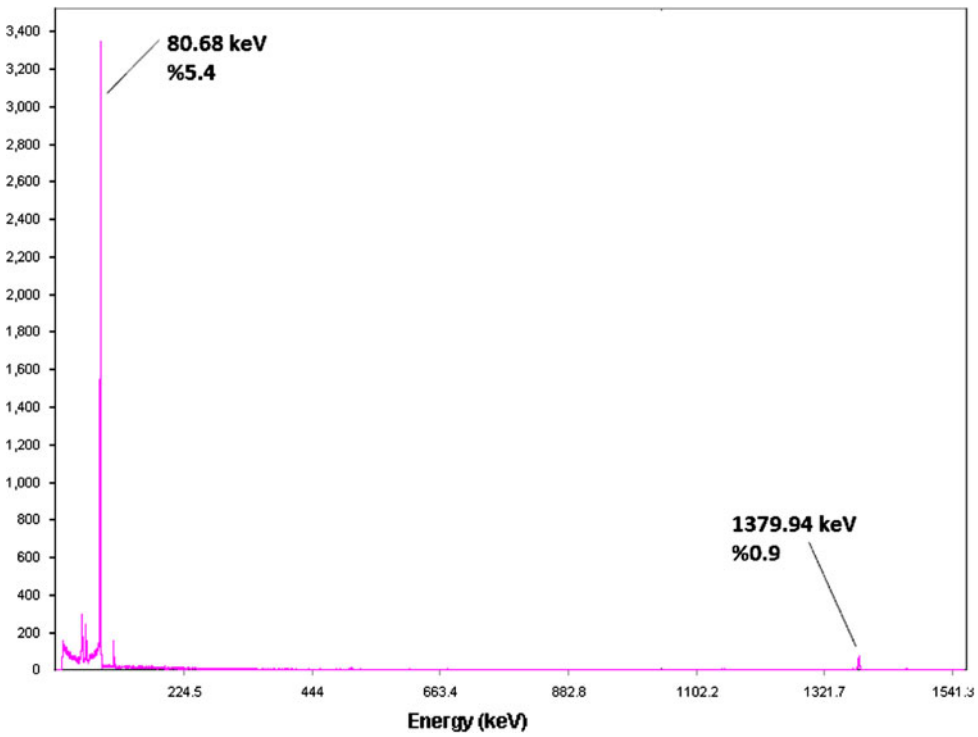

Fig. 2 Gamma spectrum for $^{166}HoCl_3$ solution used in the radiolabeling

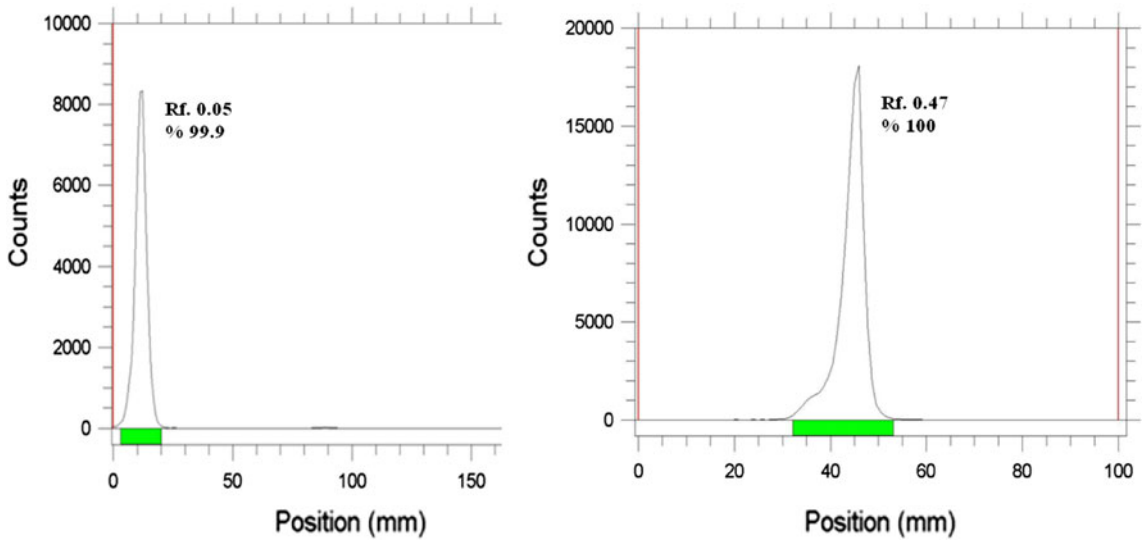

Fig. 3 ITLC of [^{166}Ho]$HoCl_3$ (*left*) and [^{166}Ho]HoTPP (*right*) in a 10 % NH_4OAc and methanol 1:1 mixture (*left*) as mobile phase on Whatman No. 2 papers

Because of the engagement of NH polar functional groups in its structure, labeling of H_2TPP with holmium cation affects its chromatographic properties and the final complex is more lipophilic. Both HPLC and ITLC data confirmed the formation of the complex. The water solubility of the radiocomplex leads to less unnecessary uptakes in tissues including liver and fat and faster kidney wash-out demonstrating the relative lipophilicity.

Radiochemical impurities in the ^{166}Ho sample used in the radiolabeling step were checked by two solvent systems; A, a mixture of 10 mM DTPA solution as mobile phase on Whatman No. 2 paper (pH 3), the free holmium cation in $^{166}Ho^{3+}$ form, was chelated with the polydentate eluting leading to the migration of the cation in ^{166}Ho-DTPA form to higher R_f (R_f 0.9), any other ionic species (such as $^{166}HoCl_4^-$, etc.) would lead to the

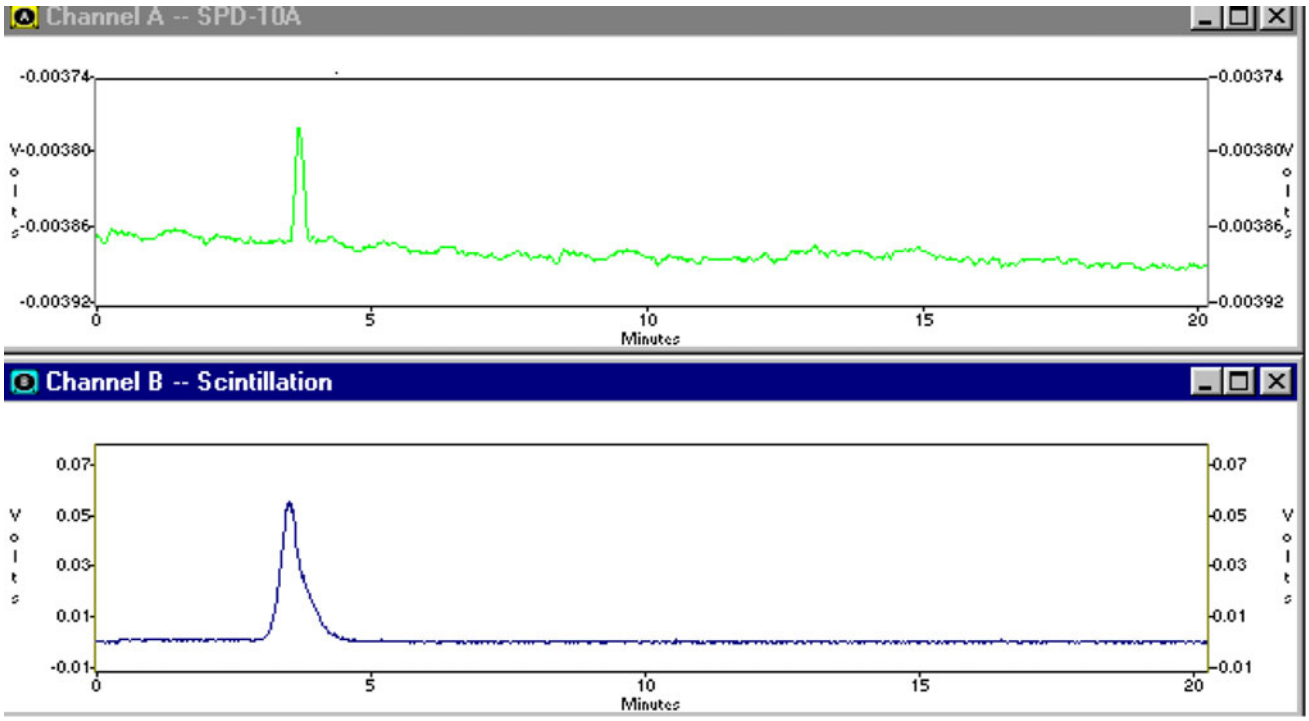

Fig. 4 HPLC chromatograms of [^{166}Ho]-TPP on a reversed phase column using acetonitrile:water 40:60, *up*; UV chromatogram, *down*; scintillation chromatogram

observation of new radiopeaks, especially at the origin (R_f 0.0–0.1).

B, a mixture of 10 % ammonium acetate:methanol (1:1) was used as another solvent system on the Whatman No. 2 paper, $^{166}Ho^{3+}$ remains at the origin using this system while other ionic species would migrate to higher R_fs (Fig. 3).

ITLC studies approved the production of a single radiolabeled compound, HPLC studies also demonstrated the existence of only one radiolabeled species using both UV and scintillation detectors. A more fast-eluting compound at 3.65 min (scintillation detector) related to 3.7 min peak (UV detector) demonstrated a more lipophilic compound compared to in cation and unlabeled compound. Free Ho-166 cation eluted at 1.22 min (not shown) (Fig. 4).

Partition coefficient of [^{166}Ho]-TPP

As expected from the chemical formula in Fig. 1, the lipophilicity of the [^{166}Ho]-TPP compound is not rather high due to the ionic nature of the radiocomplex. The measured octanol/water partition coefficient, P, for the complex was found to depend on the pH of the solution. At the pH 7 the log$P$ was 2.01.

Stability

The chemical stability of [^{166}Ho]-TPP was high enough to perform further studies. Incubation of [^{166}Ho]-TPP in freshly prepared human serum for 24 h at 37 °C showed no loss of ^{166}Ho from the complex. The radiochemical purity of complex remained at 98 % for 24 h under physiologic conditions.

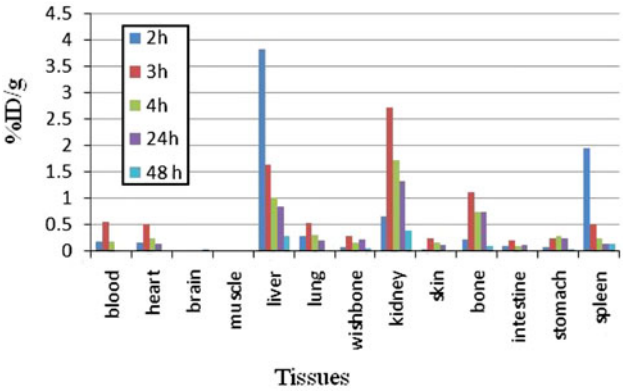

Fig. 5 Percentage of injected dose per g (%ID/g) of $^{166}HoCl_3$ in rat tissues at 2, 3, 4, 24 and 48 h post injection

Biodistribution studies

In order to investigate biodistribution of ^{166}Ho-TPP in wild-type animals we had to obtain the biodistribution data for free holmium cation in our hands, thus after injection of 6.7 MBq of the $^{166}HoCl_3$ pre-formulated by the normal

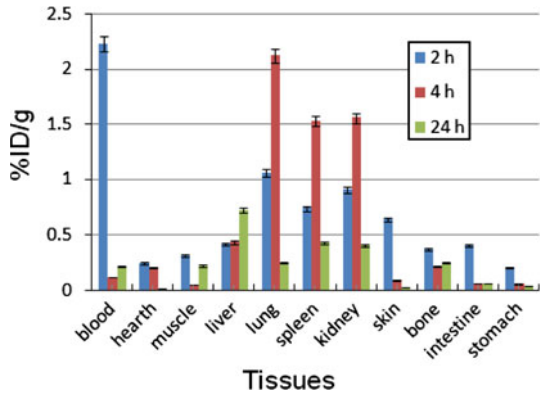

Fig. 6 Biodistribution of [^{166}Ho]-TPP (1.85 MBq, 50 μCi) in wild type rats 2, 4 and 24 h after IV injection via tail vein (%ID/g: percentage of injected dose per g of tissue calculated based on the area under curve of 80.6 keV peak in gamma spectrum) ($n = 3$)

saline (pH 6.5–7) through the tail vein of adult wild-type rats the bi distribution of the cation was checked in various vital organs.

The animals were sacrificed by CO_2 asphyxiation at selected times after injection. Dissection began by drawing blood from the aorta, followed by collecting heart, spleen, muscle, brain, bone, kidneys, liver, intestine, stomach, lung and skin samples. The tissue uptakes were calculated as the percent of area under the curve of 80.6 keV peak per g of tissue (%ID/g) (Fig. 5).

For free ^{166}Ho cation, the radioactivity was mainly located in the liver, kidney and bone. The free cation is soluble in water and it can be excreted via the urinary tract. Since the metallic ^{166}Ho is transferred in plasma into a protein-bond form, the major final accumulation was shown to be in the liver.

Due to ionic nature of the porphyrin complex, the major activity in 2 h post injection is present in blood, lung, kidney and spleen thus the major route of excretion for the labeled compound is urinary tract after 24 h. Low intestinal activity demonstrates the low hepatobiliary excretion route (Fig. 6).

For better comparison of the ^{166}Ho-TPP and $^{166}Ho^{3+}$ species behavior, Fig. 7 demonstrates the blood accumulation from 2 to 24 h. Both compounds are washed out from the circulation after 24 h, although the blood washout mechanisms are different.

Significant difference in blood activity content is observed for free Ho cation and the labeled compound, blood content is low due to rapid removal from the circulation.

Both compounds are excreted through the kidney, however in case of the labeled compound the excretion increases after 4 h significantly, while Ho-166 cation is excreted slowly in 24 h, with an almost steady manner.

As a metal cation Ho-166 has significant spleen uptake while the labeled complex is extremely accumulated in the

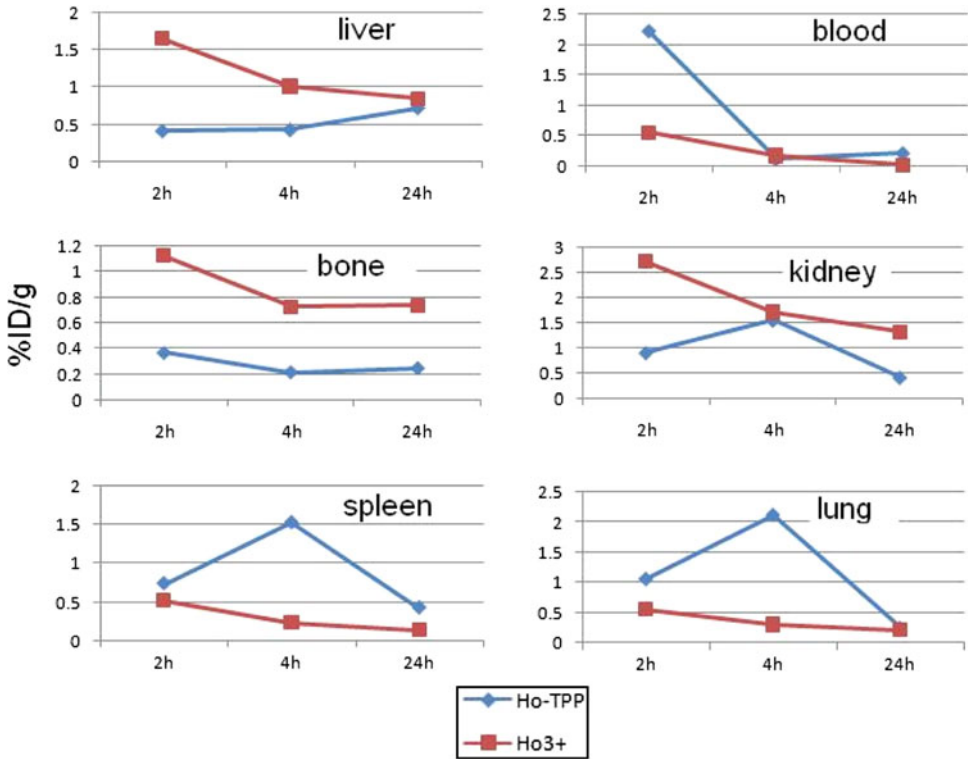

Fig. 7 Demonstrates the comparative study of vital organs uptake for ^{166}Ho-TPP and $^{166}HoCl_3$ and the kinetic pattern differences for both species. ^{166}Ho cation is accumulated in the liver in the first 24 h post injection slightly, while ^{166}Ho-TPP first major excretion route is through the liver

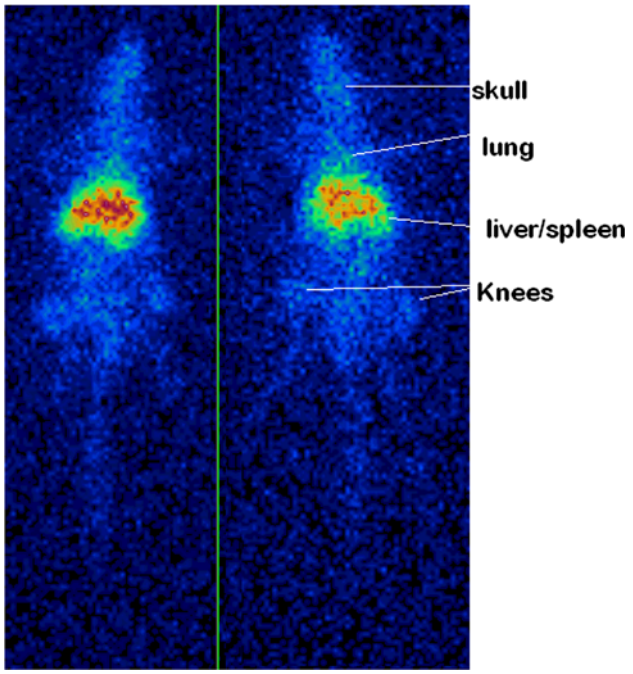

Fig. 8 SPECT images of ^{166}Ho-TPP (90 MBq, 22 μCi) in wild-type rats 8 h post injection

spleen gradually up to 1.5 % in 4 h. Both species show decreasing bone uptake in 24 h, however labeled compound uptake is much less in all time intervals.

Imaging of wild-type rats

^{166}Ho-TPP imaging in the wild-type rats showed a distinct accumulation of the labeled compound in the abdomen region and less in kidneys especially in 8 h (Fig. 8). However a small portion of activity is observed at the skeleton including skull and knees as shown by arrows. Part of upper abdominal uptake is lung accumulation as shown in the scan. Most of kidney activity is excreted from the tract leading to the fade of kidney uptake.

Conclusion

Total labeling and formulation of [^{166}Ho]-TPP took about 12 h at 50 °C (radiochemical purity: >95 ± 2 % ITLC, >99 ± 0.5 % HPLC, specific activity: 0.9–1.1 GBq/mmol). The complex was stable in final formulation and human serum for 24 h. The partition coefficient was calculated for the compound (log $P = 2.01$). A detailed comparative pharmacokinetic study performed for ^{166}Ho cation and [^{166}Ho]-TPP performed up to 24 h. The complex is mostly washed out from the circulation through kidneys and in less extends from the liver. The kidney:blood, kidney:liver and kidney:muscle ratios 4 h post injection were 14, 3.6 and 7.38 respectively. The SPECT images of the radiolabeled compound demonstrated high abdomen uptake 2–24 h post injection which is in agreement with biodistribution data. The accumulation of the tracer in other tumor models is under investigation.

Conflict of interest The authors declare no conflict of interest.

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