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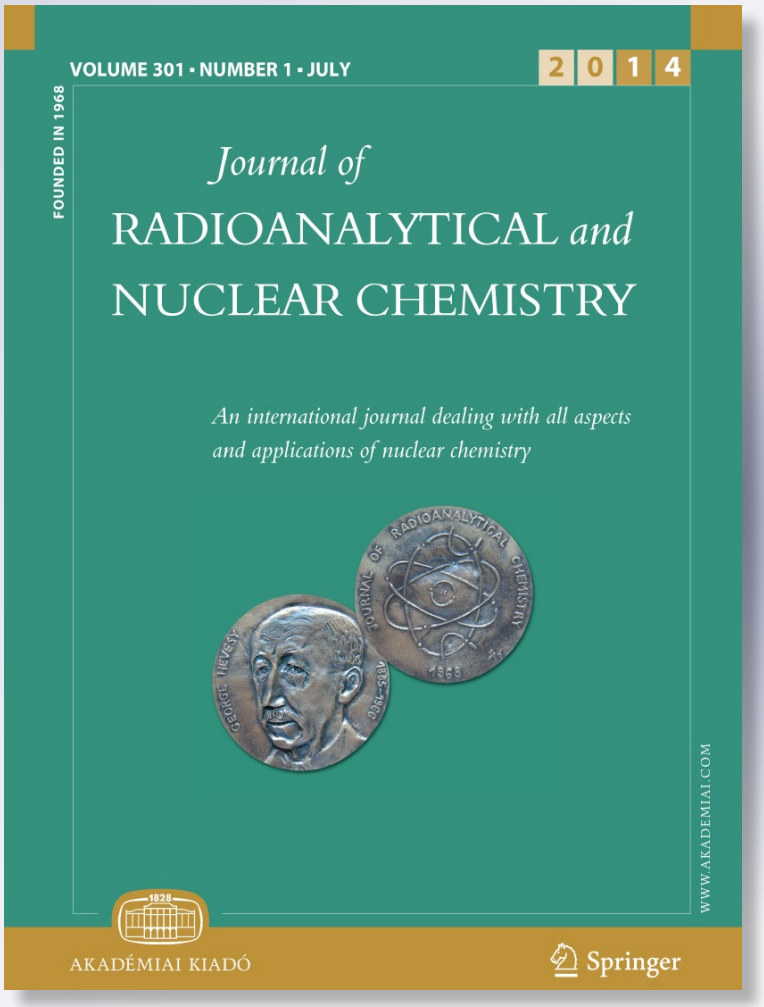

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Radiosynthesis and biological evaluation of ^{166}Ho labeled methoxylated porphyrins as possible therapeutic agents

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Abstract ^{166}Ho labeled 5,10,15,20-tetrakis(3,4-dimethoxyphenyl) porphyrin, and 5,10,15,20-tetrakis(3,4,5-trimethoxyphenyl) porphyrin ([^{166}Ho]–TDMPP and [^{166}Ho]–TTMPP respectively) were prepared with acceptable radiochemical purity and specific activities. Stability and partition coefficient of the complexes were determined in the final formulations and biodistribution studies in mouse demonstrated high accumulation of [^{166}Ho]–TDMPP in the lung and liver and less excretion through the kidney.while [^{166}Ho]–TTMPP was mostly excreted into intestines and kidneys while lungs were a minor accumulation site. In contrast to other reported radiolanthanide labeled porphyrins these two complexes showed less liver accumulation. Further investigation of their potential therapeutic properties is of interest.

Keywords Methoxylated porphyrins · ^{166}Ho · Biodistribution · Cancer therapy

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Introduction

Porphyrins have been labeled directly with therapeutic radionuclides such as ^{109}Pd [1, 2], ^{188}Re [3] and ^{166}Ho [4] for therapeutic purposes and due to the sub-optimal biodistribution and diverse physical properties of these radionuclides, further investigations on the development of these potential therapeutic tumor targeting agents is still ongoing. Therefore, few conjugated porphyrin molecules have been developed and radiolabeled with therapeutic radio nuclides including ^{177}Lu–DOTA porphyrins [5] and ^{90}Y–DOTA porphyrins [6] . In order to obtain radiocomplexes with higher stability.

Sufficient activity levels of many beta-emitters such as ^{153}Sm, ^{177}Lu, ^{188}Re and ^{166}Ho can be produced using direct neutron capture (n,γ) reaction. ^{166}Ho (E_{β^-} max = 1.84 - MeV, $T_{1/2}$ = 26.8 h) is a readily available and an interesting radionuclide for targeted therapy. Although it is not available in high specific activity, the uni-elemental ^{166}Ho makes it an accessible and inexpensive radionuclide and the obtained specific activity is sufficient for radiolabeling of small molecules.

No-carrier-added ^{166}Ho is also available from decay of reactor-produced ^{166}Dy, and the efficacy of the ^{166}Dy/^{166}Ho in vivo generator system [7], for production of ^{166}Ho and the radiolabeling of various ligands for possible targeted therapy applications, such as ^{166}Dy/^{166}Ho–biotin for tumor targeting macroaggregates in radiation synovectomy has been established [8]. It has been reported that the stability of the daughter complex is not significantly lower than that of the parent ^{166}Dy complex.

Due to the interesting pharmacological properties of porphyrins such as solubility in serum, rapid blood washout, tumor avidity and feasible complexation with various bi/tri-valent metals [9], the idea of developing possible

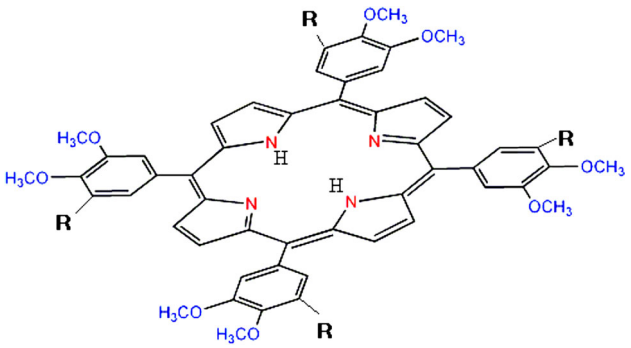

Fig. 1 Structures of H_2TDMPP (R=H) and H_2 TTMPP (R=OCH_3)

tumor targeting agents for therapeutic applications by incorporating ^{166}Ho into various suitable porphyrin ligands has been investigated. Recently tetraphenyl porphyrin (TPP) ligand was used for radiolabeling using various radionuclides including ^{166}Ho [4], however, high lipophilicity of the complex led to high liver uptakes, the idea of developing new more lipophilic radiolabeled porphyrins with potential metabolic handle sites compared to TPP leading to greater water solubility and/or diversity in excretion routes was of great interest. Two methoxylated porphyrins; H_2TDMPP and H_2TTMPP (Fig. 1), were considered interesting ligands due to the possibility of metabolism via –OCH_3 demethylation leading to the more water soluble species as well as better conjugation handles for excretion.

In this work we report the synthesis, radiolabeling, quality control, stability, partition coefficient determination and biodistribution studies of ^{166}Ho–TDMPP and ^{166}Ho–TTMPP mouse.

Experimental

Production of ^{166}Ho was performed at the Tehran Research Reactor (TRR) using the ^{165}Ho(n,γ)^{166}Ho nuclear reaction. Natural holmium nitrate with purity of >99.99 % was obtained from ISOTEC Inc. Chemical components were obtained from Sigma-Aldrich Chemical Co., UK. Calculations were based on the 80.6 keV photon peak for ^{166}Ho. Beta spectroscopy was carried out using the Wallac 1220 Quantulus liquid scintillation spectrometer. NMR spectra were obtained on a FT-80 Varian instrument (80 MHz) with tetramethylsilane as the internal standard. Mass spectra were recorded by a Finnigan Mat TSQ-70 Spectrometer. All buffer and solvents used for labeling were of high purity and filtered through 0.22 μm Cativex filters. Paper chromatography was performed by counting Whatman No. 2 papers using a thin layer chromatography scanner, Bioscan AR2000, Bioscan Europe Ltd. (France). Analytical high performance liquid chromatography (HPLC) used 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, NJ (USA). A standard curve was generated to calculate the mass of the final solution. Biodistribution data were acquired by counting normal saline washed tissues after weighing using a Canberra^{TM} high purity germanium (HPGe) detector (model GC1020-7500SL). Radionuclidic purity was checked with the same detector. For measurement of the activity of the samples a CRC Capintech Radiometer (NJ, USA) was used. Animal studies were performed in accordance with the United Kingdom Biological Council's Guidelines on the Use of Living Animals in Scientific Investigations, 2nd ed.

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

^{166}Ho 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 [10] at the Tehran Research Reactor at a thermal neutron flux of 4×10^{13} n/cm^2/s. 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 filter (Millipore, Millex GV) for use in radiolabeling. 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)).

Preparation of 5,10,15,20-tetrakis(3,4-dimethoxyphenyl) porphyrin (H_2TDMPP) and 5,10,15,20-tetrakis(3,4,5-trimethoxyphenyl) porphyrin (H_2TTMPP)

The compounds were prepared according to the reported method using freshly distilled requisite methoxylated benzaldehydes, pyrrole and propionic acid followed by oxidation [11].

Spectral analyses confirmed the structure of the prepared. H_2TDMPP: UV (CH_2Cl_2) λ_{max} = 424, 520, 556, 594, 650 nm. ^{1}H NMR (DMSO-d_6): −2.7 (NH), 8.93 (H-pyrrole), 7.80, 7.79, 7.78 (H_{o1},H_{o2}), 7.29, 7.28(H_m), 4.20, 3.97($H_{por}Ho_{OMe}$). Mass (*m/e*) 558 (M+3).

H_2TTMPP: m.p. 128 °C; UV (CH_2Cl_2) λ_{max} 424 nm. ^{1}H-NMR (DMSO-d_6) 3.58 (s, 24H, 8OCH_3), 3.74 (s, 12H, 4OCH_3), 4.00 (s, 1H, NH), 5.22 (d, 2H, 2 pyrrolic CH), 5.61 (d, 2H, 2 pyrrolic CH), 5.87 (d, 2H, 2 pyrrolic CH), 6.54 (d, 4H, Ar–H), 6.61 (d, 4H, Ar–H), 7.91 (d, 2H, 2 pyrrolic CH), 10.44 (s, 1H, NH); Mass (*m/e*) 673 (M+3).

Preparation of ^{166}Ho-labeled porphyrins

The acidic solution (2 mL) of holmium chloride (37–45 MBq, 1–1.25 mCi) were transferred to separate 3 mL borosilicate vials and heated to dryness using a flow of N_2 gas at 50–60 °C. Fifty microlitres of porphyrin solutions in dichloromethane (5 mg/mL) (equivalent to H_2TDMPP; 4–5 μmole, H_2TTMPP; 1.5 μmole) were added to the holmium-containing vials followed by the addition of acetate buffer pH 5.5 (1,000 μL). The mixture was refluxed at 80 °C and subjected to chromatographic tests (paper chromatography and HPLC) up to 24 h. The active solution reaching 99 % of radiochemical purity was then passed through a 0.22 μm filter and pH was adjusted to 5.5–7.

Quality control of ^{166}Ho-labeled porphyrins

Radio thin layer chromatography

A 5 μL sample of the final fractions were spotted on Whatman No. 2 chromatography paper, and developed in the mobile phase mixture, 10 % NH_4OAc and methanol 1:1.

High performance liquid chromatography

HPLC experiments were performed with a flow rate of 1 mL/min, pressure: 130 kgF/cm^2 for 20 min 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 coefficients

Partition coefficient (log *P*) of each labeled porphyrin was calculated by determination of ratio of 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 ^{166}Ho-radiolabeled 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

In final solution

The stability of the complexes was checked according to the conventional paper chromatography method [12]. A sample of radiolabeled complex (37 MBq) was kept at room temperature for 24 h while being checked by paper chromatography at time intervals in order to check stability in final product using the above chromatography system.

In presence of human serum

For serum stability studies, 300 μL of freshly prepared healthy human serum was added to 7.4 MBq (200 μCi, 100 μL) of final solution of radiolabeled complex and the resulting mixture was incubated at 37 °C for 24 h. Every 4 h to a portion of the 50 μL of the mixture was added, trichloroacetic acid (10 %, 100 μL) and the resulting mixture was centrifuged at 3,000 rpm for 5 min followed by decanting the supernatant from the debris. The stability was determined by paper chromatography analysis of supernatant using above mentioned TLC system.

Biodistribution in wild-type mice

The distributions of the radiolabeled complexes as well as the free $^{166}Ho^{3+}$ among tissues were determined in mouse. 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, the tissues (blood, heart, lung, brain, intestine, feces, skin, stomach, kidneys, liver, muscle and bone) were weighed and rinsed with normal saline and their radioactivity per gram of tissue were determined with an HPGe detector equipped with a sample holder device as percent of injected dose per gram of tissues. Blood samples were rapidly taken from rodent aorta after anesthesia.

Results and discussion

Methoxylated porphyrin

During in vitro studies many groups have demonstrated that meta –OMe porphyrin isomers are ~10-fold more lipophilic than corresponding ortho substituted compounds, which enhances their cellular uptake and allows

accumulation in mitochondria (relative to cytosol) and crossing the blood–brain barrier to a much higher extent potentially leading to more tumor cell uptake due to increased mitochondrial accumulation [13]. On the other hand phenyl porphyrins have demonstrated high liver accumulation leading to more radiation exposure [3]. Therefore, the development of methoxylated porphyrins seemed logical due to the better penetration as well as polarity of possible metabolites after demethylation.

In continuation of our recent work in this field [4, 14–16], we were interested in preparation and radiolabeling of a new series of methoxylated porphyrins for ultimate biodistribution studies in animal tumor models. The porphyrins were prepared according to reported methods with slight modifications and were re-purified by repeated column chromatography.

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 of 3–5 MBq/mg for radiolabeling use. After counting the samples on an HPGe detector for 5 min, two major photons (5.4 % of 80.68 keV and 0.9 % of 1379.94 keV) were observed (Fig. 2). The ^{166}Ho 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 filtration.

Determination of radiochemical purity was performed in 10 mmol/L DTPA aq. solution (solvent 1). In this solvent free Ho^{3+} cation is converted to more lipophilic HoDTPA form and migrates with a higher R_f. A small radioactive fraction remaining at the origin could be related to other Ho ionic species, not forming the HoDTPA complex, such as $HoCl_4^-$, etc. and/or colloids. On the other hand, 10 % ammonium acetate:methanol mixture (1:1) (solvent 2) was also used for the determination of radiochemical purity.

In this system, other Ho ionic species (such as $HoClO_4^-$, etc.) migrate to higher R_f (not detected) and free Ho^{3+} cation as well as colloids retain at the origin (R_f 0.0) as shown in Fig. 3, the only detected impurity seems to be <1 % colloids.

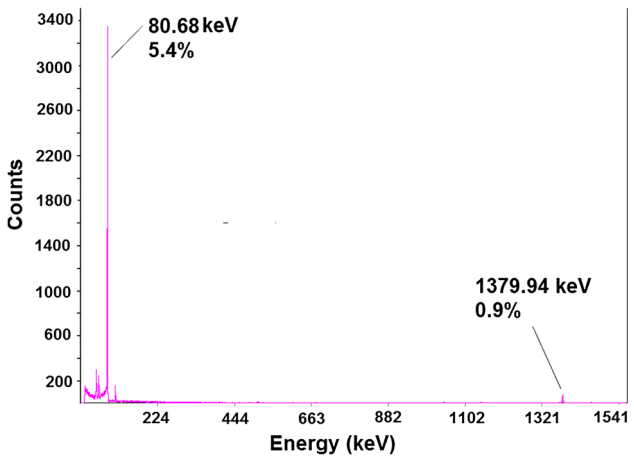

Fig. 2 Gamma spectrum of $^{166}HoCl_3$ solution used in the radiolabeling

Radiolabeling

Because of the engagement of the two NH polar functional groups in its structure (Fig. 1), labeling of porphyrins with the holmium trivalent cation affects chromatographic properties by decrease in polarity and the final complex is possibly more lipophilic.

Radiochemical purity of the final complex dissolved in aqueous medium was checked by paper chromatography using a mixture of 10 % ammonium acetate:methanol (1:1) as mobile phase and a silica-gel sheet (10 × 15 cm). Figure 4 illustrates the radiochromatograms for ^{166}Ho–TDMPP and ^{166}Ho–TTMPP under the conditions described (R_f. 0.8 for ^{166}Ho–TDMPP and R_f. 0.85 for ^{166}Ho–TTMPP). The small difference between R_f values is due to the slightly higher lipophilicity of the tri-methoxy compound compared to the di-methoxy complex.

Although the paper chromatography studies demonstrated the production of the radiolabeled compound, HPLC studies using both UV and scintillation detectors demonstrated the existence of radiolabeled species (Fig. 5).

For ^{166}Ho–TDMPP a faster eluting major peak at 3.72 min (scintillation detector) related to 3.62 min peak (UV detector, not shown) demonstrated more hydrophilic compound. Free Ho cation usually was eluted at 1.62 min which was not detected in the sample suggesting high radiochemical purity (>99 %).

As expected, both methoxylated porphyrin samples are eluted later than the free cation. The difference among the retention times of the two samples (3.72 min for [^{166}Ho]–TDMPP and 4.31 for [^{166}Ho]–TTMPP is in agreement with their lipophylicities the more lipophilic trimethoxy complex is eluted later than the other complex.

Partition coefficients of radioholmium complexes

As expected from their chemical behaviors, the lipophilicities of the methoxylated labeled compounds is significant due to the presence of methoxy groups. The measured octanol/water partition coefficients, P, for the complexes were found to depend on the pH of the solution. At the pH 7 the log P for [^{166}Ho]–TDMPP and [^{166}Ho]–TTMPP were 1.00 and 1.26, respectively. The tri-methoxy complex demonstrated higher log P due to greater lipophilicity.

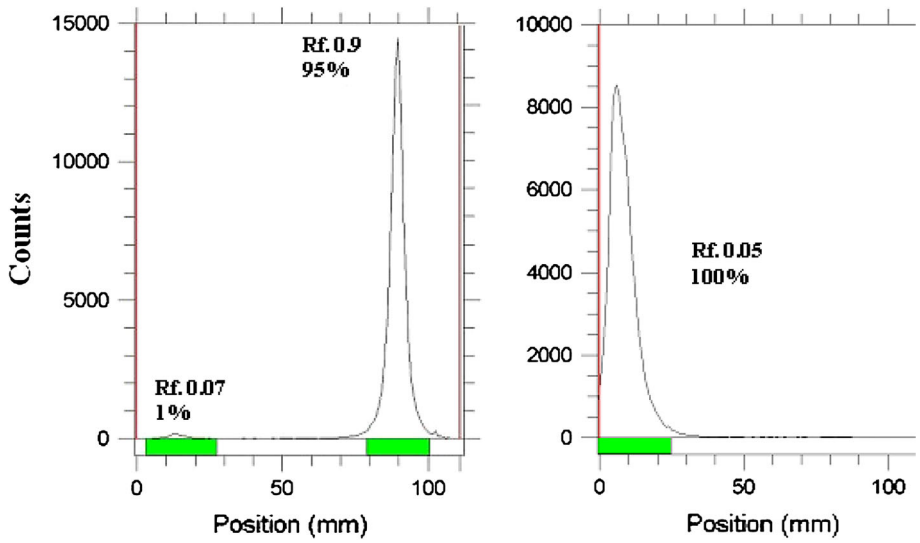

Fig. 3 ITLC chromatograms of $^{166}HoCl_3$ solution in 10 mM DTPA solution (pH ~ 4) (*left*) and in 10 % ammonium acetate:methanol (1:1) (*right*) on Whatman No. 1 paper

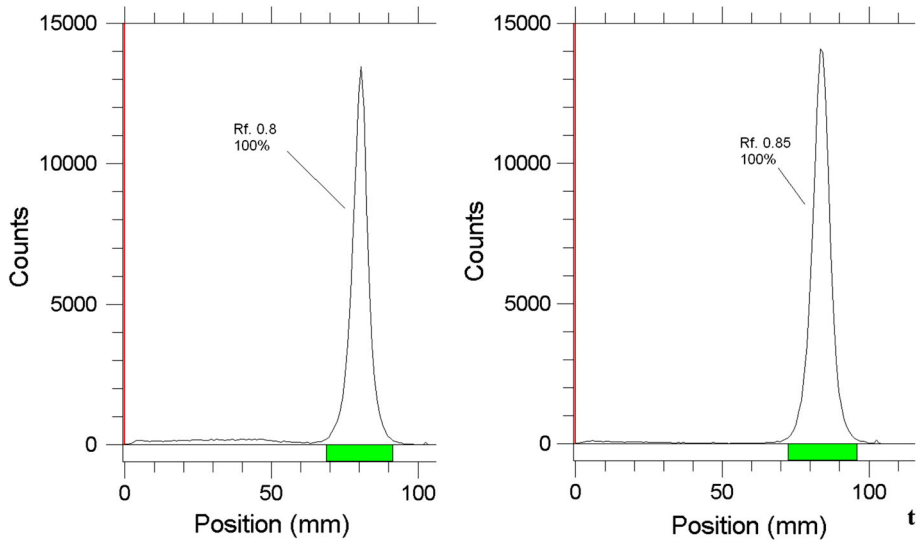

Fig. 4 Paper chromatograms of ^{166}Ho–TDMPP (*left*) and ^{166}Ho–TTMPP (*right*) in a 10 % NH_4OAc and methanol 1:1 mixture as mobile phase on

Stability

The chemical stability of radiolanthanide complexes were high and incubation of the ^{166}Ho-complexes in freshly prepared human serum for 24 h at 37 °C showed no loss of ^{166}Ho from the complex. The radiochemical purity of the complexes did not decrease for 24 h under physiological conditions.

Biodistribution studies

The animals were sacrificed by CO_2 asphyxiation at selected time points after tracer injection (2, 4 and 24 h for the cation and 4, 24 and 72 h for complexes). Dissection began by drawing blood from the aorta followed by removing the heart, spleen, muscle, bone, kidneys, liver, intestine, stomach, lungs and skin samples. The tissue uptake values were calculated as the percent of area under the curve of the related photo peak per gram of tissue (% ID/g) (Fig. 6).

Free $^{166}Ho^{3+}$ cation

The liver uptake of the cation is comparable with many other radio-lanthanides mimicking calcium cation accumulation and about 0.7 % of the activity accumulates in the liver in 24 h. The major excretion site is urinary route as observed for Ca^{2+} cation. Also bone uptake is significant esp. in 24 h and remains constant up to 24 h.

[^{166}Ho]–TDMPP complex

The radiolabeled compounds biodistribution is also demonstrated in Fig. 7. In comparison to our recently reported

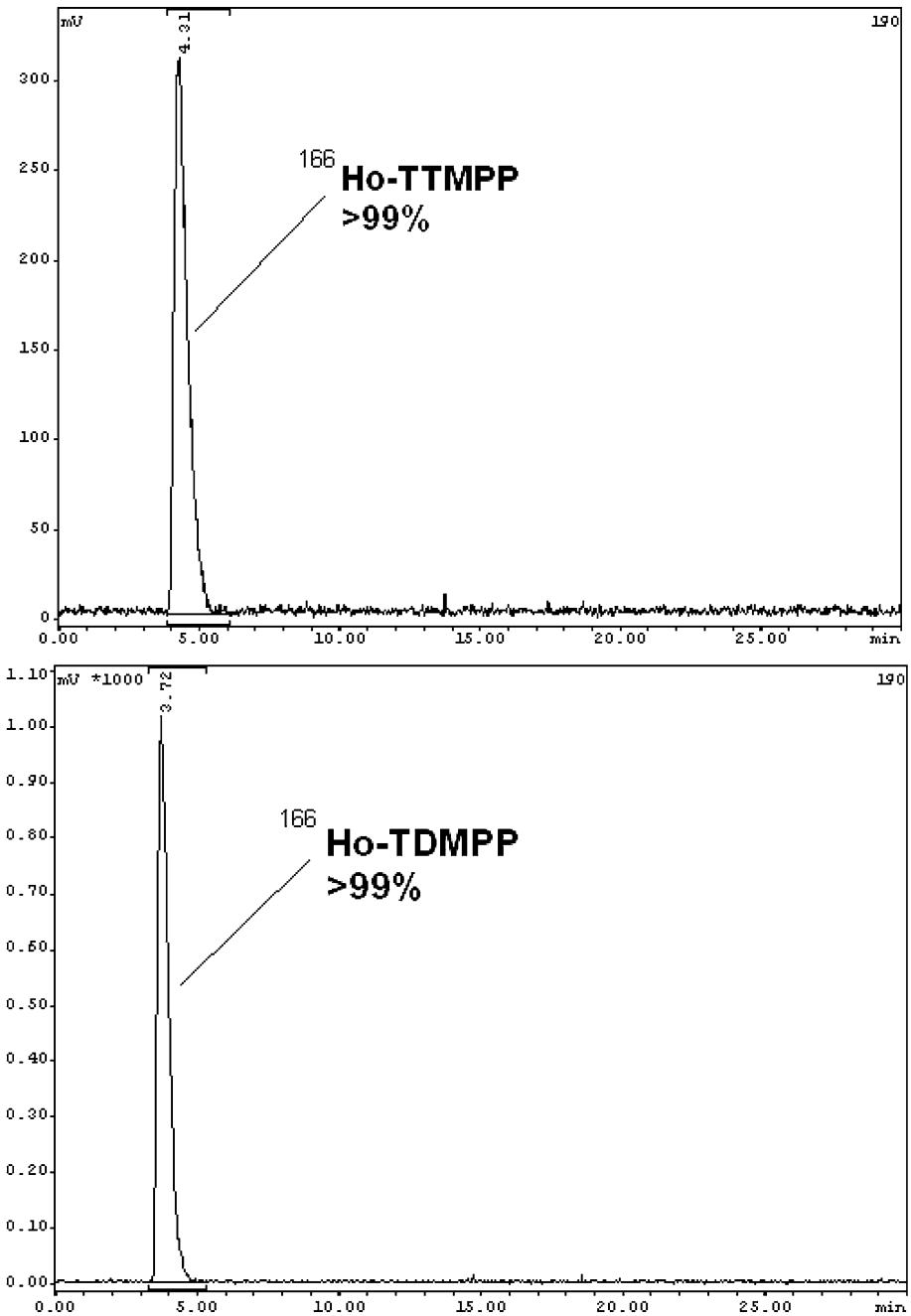

Fig. 5 HPLC chromatograms for [^{166}Ho]–TDMPP (*below*) [^{166}Ho]–TTMPP (*up*) on a reversed phase column using acetonitrile:water 40:60

similar radiolholmium labeled porphyrin i.e. [^{166}Ho] labeled 5,10,15,20-tetrakis(phenyl) porphyrin [4], ^{166}Ho–TDMPP possess higher liver and lung uptakes showing more infiltration into cells. Interestingly, in another recent study, ^{166}Ho and ^{153}Sm-porphyrins showed significant lung uptake [14, 15]. The actual mechanism of lung uptake is not understood however, it can be proposed that dismutase enzyme system can lead to ^{166}Ho release from the complex into the cells. Significant liver uptake is possibly due to natural liver uptake of porphyrins after attachment to serum lipoproteins and/or their lipophylicities. However, a small fraction of the complex is washed out through the kidneys up to 4 h, which might be a result of more water soluble metabolite(s), compared to other reported porphyrins with significant urinary excretion [14, 16, 17].

[^{166}Ho]–TTMPP complex

TTHMP complex is distributed differently from TDMPP, since the major fraction is excreted through the intestines. The expression of proteins that possibly affect porphyrin accumulation including ferrochelatase and ATP-binding

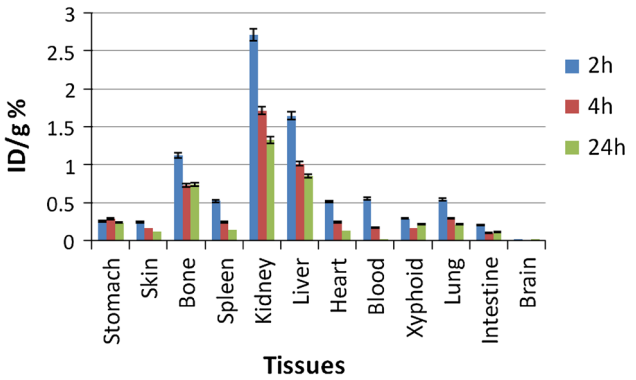

Fig. 6 Percentage of injected dose per gram (ID/g%) of $^{166}HoCl_3$ in rat tissues at 2, 4 and 24 h post injection

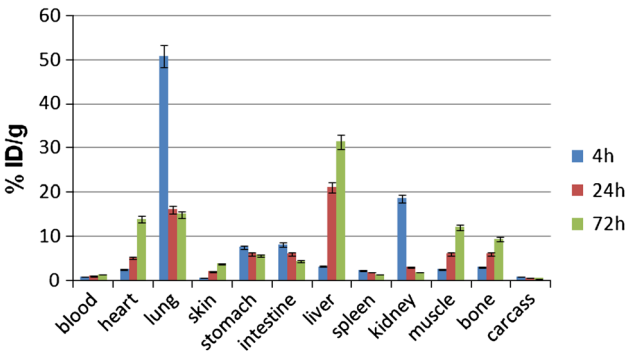

Fig. 7 Biodistribution of [^{166}Ho]–TDMPP (1.85 MBq, 50 μCi) in wild type mice after iv injection via a lateral tail vein (ID/g%: percentage of injected dose per gram of tissue, $n = 3$)

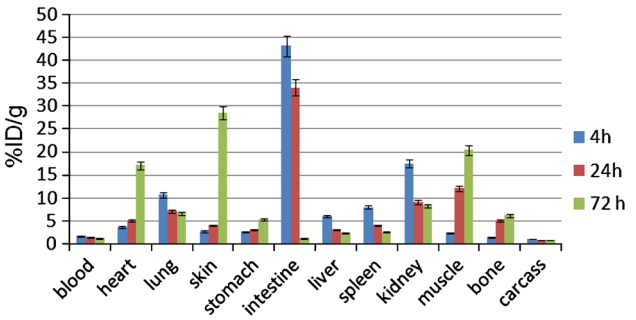

Fig. 8 Biodistribution of [^{166}Ho]–TTMPP (1.85 MBq, 50 μCi) in wild type mice after iv injection via a lateral tail vein (ID/g%: percentage of injected dose per gram of tissue, $n = 3$)

cassette transporter G2 (ABCG2), in several tumor cell lines as well as normal tissues has been reported. The complexes are distributed not only in the plasma membrane but also intracellular organelles, including mitochondria. Thus mitochondria-rich organs such as liver and myocardial cells can uptake radiolabeled porphyrins followed by the release of the cation in mitochondrion matrix [18]. Thus the various uptakes of the labeled compounds in heart, liver and even lungs can be an outcome of above mentioned mechanism as already reported for other radiolabeled TDMPP analogs [13, 19] (Fig. 8).

The biodistribution of the two radiolabeled TDMPP complexes differ significantly from each other and this might be due to the formation of different complex structures despite high similarities among the two structures. It can be proposed the different complexes would enter different metabolic pathways leading to compounds with different distributions.

Conclusion

[^{166}Ho]–TDMPP and [^{166}Ho]–TTMPP complexes were prepared with acceptable radiochemical purity (>99.9 %; paper chromatography and >99.9 %; HPLC) and significant specific activities (9–10 GBq/mmol; [^{166}Ho]–TDMPP and 29–30 GBq/mmol; [^{166}Ho]–TTMPP) which were shown to be lipophilic species (log $P = 1.00$ and log $P = 1.26$, respectively). The [^{166}Ho]–TDMPP complex is mostly accumulated in the lung and liver and less excreted through the kidneys. Liver also demonstrated significant activity uptake in 72 h post injection due to the natural LDL-binding of porphyrins. [^{166}Ho]–TTMPP, is mostly excreted into intestines and kidneys and lungs are the minor accumulation sites. It is proposed that increasing the lipophilicity by developing methoxylated porphyrins resulted in, higher mitochondrial uptake was observed which can lead to higher tumor cell uptake with overexpressed mitochondrial organelles. Further investigations on their therapeutic properties will be conducted.

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