

**Development of  $^{166}\text{Ho}$ -zoledronate as a bone marrow ablative agent**

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**Abstract**

In this study, production, quality control and biodistribution studies of  $^{166}\text{Ho}$ -zoledronate have been presented as a possible bone marrow ablative agent. Ho-166 chloride was produced by thermal neutron irradiation of natural  $^{165}\text{Ho}(\text{NO}_3)_3$  samples.  $^{166}\text{Ho}$ -zoledronate complex was prepared by adding the desired amount of zoledronate solution (0.2 mL, 150 mg/ml in 1 M NaOH) to appropriate amount of the  $^{166}\text{HoCl}_3$  solution. Radiochemical purity of the complex was monitored by instant thin layer chromatography (ITLC). Stability studies of the complex in the final preparation and in the presence of human serum were performed up to 48 h. The biodistribution of  $^{166}\text{Ho}$ -zoledronate and  $^{166}\text{HoCl}_3$  in wild-type mice was checked up to 72 h.  $^{166}\text{Ho}$ -zoledronate complex was prepared in high radiochemical purity (> 99%, ITLC) and specific activity of 4.4 GBq/mmol. The major accumulation of radiolabelled complex was observed in the bone tissue. These findings suggest  $^{166}\text{Ho}$ -zoledronate has can be a possible candidate for bone marrow ablation in patients with multiple myeloma.

**Keywords:** Bone marrow ablation, Ho-166, Zoledronate, biodistribution

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**Introduction**

Over the past 30 years, bisphosphonates were established as an effective new drug class for bone pain palliation in patients with different complicated malignancies such as prostate, breast and lung cancer (1). Bisphosphonates have strong affinity for calcium phosphates and for hydroxyapatite and attach preferentially to bone with increased avidity for areas of osteoblastic and osteoclastic activity (2,3).

Nowadays, researches have shown that skeletal targeted radiotherapy in patients

with multiple myeloma can improve the response rate in phase I and II trials, with promising long-term survival data (4). Several bone-seeking radiopharmaceuticals have been developed for bone marrow ablation in multiple myeloma and other haematological malignancies (5).

Samarium-153-ethylene diamine tetramethylene phosphonate (153Sm-EDTMP) is now considered as an efficient radiopharmaceutical for bone pain palliation (6). Various other radiopharmaceuticals such as rhenium-

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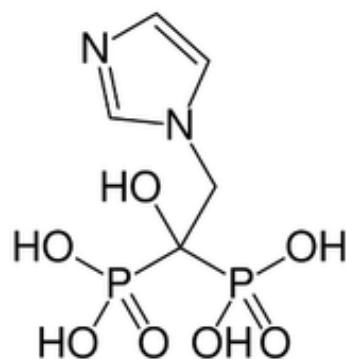
186-hydroxyethylidenediphosphonic acid (186Re-HEDP) (7),  $^{166}\text{Ho}$ -1,4,7,10-tetraazacyclododecane-1,4,7,10-tetramethylene-phosphonate ( $^{166}\text{Ho}$ -DOTMP) (8) and Tin-117m (9) have also been investigated for targeted radiotherapy of bone malignancies.

In order to give sufficient dose to the bone marrow, radionuclides with higher beta particle energy are recommended for bone marrow ablation.  $^{166}\text{Ho}$  with a half-life of 26.8 h decays with two principal  $\beta^-$  emissions (1.85 MeV (51%) and 1.77 MeV (48%)), and three photon energies (80.58 MeV (6.56%), 1379.44 (0.92%) and 1581.83(0.18%)) (10) is an excellent radionuclide for bone marrow ablation due to the high energy of the  $\beta^-$  particles. The short physical half-life permits delivery of high-dose irradiation to peripheral blood stem cells during chemotherapy and reinfusion of cryopreserved within 6-10 d (8).

Various therapeutic possible  $^{166}\text{Ho}$  bone-seeking agents have been reported such as  $^{166}\text{Ho}$ -DOTMP (8),  $^{166}\text{Ho}$ -EDTMP (5) and  $^{166}\text{Ho}$ -APDDMP (11). The presence of extra amounts of bisphosphonate ligand in the final formulation is inevitable in all mentioned agents which can impose toxicity concerns especially for non-pharmaceutical ligands, however the use of a pharmaceutical ligand such as zoledronate with known toxic dose and biological behaviour and availability can be a superiority. However, the search for the development of new  $^{166}\text{Ho}$ -bisphosphonate ligands with higher stability, better pharmacokinetics and lower unwanted tissue uptakes (liver and gasterointestinal tract) is still ongoing. There are two separate categories for beta emitters used in clinics, i.e. bone pain palliative and bone ablative agents. The difference is mostly based on the range

of beta particle traveling in the tissues. For instance Sm-153 has a lower range and usually does not reach the bone marrow tissue and is just reducing the bone proliferative cancerous cells, while  $\text{Ho-166}$  with longer range of beta emission is really affecting the bone marrow and its radiopharmaceuticals are intended for irradiation of bone marrow tissue in bone marrow cancers.

A number of bisphosphonates such as clodronate, etidronate, pamidronate, zoledronate and ibandronate are commercially available in some countries. Among the mentioned bisphosphonates, zoledronate (Fig. 1) is a potent agent which inhibits osteoclast-mediated bone-resorption (12). In inhibiting the bone-resorption, zoledronate is approximately 1,000 times stronger than etidronate (13). Moreover, the binding affinity of zoledronate for bone is 10 orders of magnitude higher than pamidronate (14).



**Figure 1** Chemical structure of zoledronic acid

Treatment of post-menopausal women with zoledronate has demonstrated normalization of the rate of bone turnover, significant increase bone mineral density of the spine, hip, wrist and total body, and significant reductions in the risk of vertebral

(spine) fractures, wrist fractures, hip fractures, and all non-vertebral fractures (15). In this work, <sup>166</sup>Ho-zoledronate was prepared as a new complex for bone marrow ablation and biodistribution studies of this complex was investigated in wild-type mice up to 72 h.

## **Materials and methods**

### *Materials and instruments*

Natural holmium nitrate with purity of >99.99% was obtained from ISOTEC Inc (USA). Production of <sup>166</sup>Ho was performed using <sup>165</sup>Ho(n, $\gamma$ )<sup>166</sup>Ho nuclear reaction in Tehran Research Reactor (5 MW). The precursor zoledronate hydrochloride with Pharmaceutical grade was a gift from Sobhan Co., Tehran Iran, with > 99% purity. Radio-chromatography was performed by counting of ITLC-SG and/or Whatman papers using a thin layer chromatography scanner, Bioscan AR2000 (Paris, France). ITLC-SG was purchased from UK. Other chemicals were purchased from Sigma-Aldrich. A high purity germanium (HPGe) detector coupled with a Canberra™ (model GC1020-7500SL) multichannel analyzer and a dose calibrator ISOMED 1010 (Dresden, Germany) were used for counting distributed activity in mice organs. Calculations were performed based on the 80.6 keV peak for <sup>166</sup>Ho. Animal studies were performed in accordance with the United Kingdom Biological Council's Guidelines on the Use of Living Animals in Scientific Investigations, 2nd edn. The wild-type mice weighing between 20-25 g were acclimatized at proper rodent diet .

### *Production and quality control of <sup>166</sup>HoCl<sub>3</sub> solution*

The desired amount (about 100 µg) of natural <sup>165</sup>Ho(NO<sub>3</sub>)<sub>3</sub> was irradiated at a thermal neutron flux of  $4 \times 10^{13}$  n.cm<sup>-2</sup>.s<sup>-1</sup>. The irradiated target was dissolved in 200 µl of 1.0 M HCl, to prepare <sup>166</sup>HoCl<sub>3</sub>. The solution was filtered through a 0.22 µm filter (Millipore, Millex GV, USA). The radionuclidic purity of the solution was checked using beta liquid scintillation as well as HPGe spectroscopy. Also the radiochemical purity of the <sup>166</sup>HoCl<sub>3</sub> was studied using instant thin layer chromatography (ITLC) method.

### *Radiolabeling of zoledronate with <sup>166</sup>HoCl<sub>3</sub>*

A stock solution (50 mL) of zoledronate was prepared by dissolving in 1 M NaOH and diluted to the appropriate volume with ultra-pure water, in order to produce a solution of 20 mg/ml. For labelling, an appropriate amount of the <sup>166</sup>HoCl<sub>3</sub> solution, containing the required activity was added to the desired amount of zoledronate solution (0.2 mL). The complex was maintained at 40°C for 2 hours. The final solution was passed through a 0.22 µm membrane filter and the pH was adjusted to 7-8.5 with 0.05 M phosphate buffer (pH 5.5). The radiochemical purity was determined using Whatman 3 MM chromatography paper or ITLC-SG, eluted with NH<sub>4</sub>OH (56%): methanol (100%): water (100%) (0.2:2:4; v/v/v) mixture.

### *Stability studies*

The stability of the complexes stored at room temperature (25 °C ambient) and in human serum at 37 °C at different time intervals were checked by determining the radiochemical purity of the complex by paper chromatography

in NH<sub>4</sub>OH:MeOH:H<sub>2</sub>O (2:20:40) system.

*In vitro stability of <sup>166</sup>Ho-zoledronate in presence of human serum*

Final solution (200 µCi, 50 µL) was incubated in the presence of freshly prepared human serum (300 µL) (Purchased from Iranian Blood Transfusion Organization, Tehran, Iran) and kept at 37 °C for 2 h. Trichloroacetic acid (10%, 100 µL) was added to a portion of the mixture (50 µL) every 30 min and the mixture was centrifuged at 3000 rpm for 5 min. The debris was decanted from the supernatant. The stability was determined by performing frequent ITLC analysis of supernatant using above mentioned ITLC system.

*Hydroxyapatite binding assay*

The hydroxyapatite binding assay was performed according to the procedure described previously (16), with only a slight modification. In brief, to vials containing 1.0, 2.0, 5.0, 10.0, 20.0 and 50.0 mg of solid hydroxyapatite, 2mL of saline solution of pH 7.4 were added and the mixtures were shaken for 1h. Then, 50 µL of the radioactive preparation (containing 100-300 µCi) was added and the mixtures were shaken for 24 h at room temperature. The suspensions were centrifuged, and two aliquots of the supernatant liquid were taken from each vial and the radioactivity was measured with a well-type counter. Test experiments were performed using a similar procedure, but in the absence of hydroxyapatite. The percentage binding of <sup>166</sup>Ho-labeled compound to hydroxyapatite (HA) was calculated according to HB=1-A/B×100, where A is the mean radioactivity value of the supernatant sample under study

and B is the mean total value of whole activity used.

*Biodistribution of <sup>166</sup>Ho cation and <sup>166</sup>Ho-zoledronate in wild-type mice*

50-100 µL of <sup>166</sup>HoCl<sub>3</sub> and final <sup>166</sup>Ho-zoledronate solution with 200 µCi radioactivities was injected intravenously to mice through their tail vein. The animals were sacrificed at the exact time intervals (2, 4, 24, 48, 72h) post injection the percent injected dose per gram (%ID/g) of different organs was calculated based on the measurements of HPGe detector. Three animals were used for each time interval.

## Results

*Radionuclide production*

The radionuclide was prepared in a research reactor with a range of specific activity between 3 to 5 GBq/mg. 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. Radionuclide purity was higher than 99.96%. The radiochemical purity of the <sup>166</sup>HoCl<sub>3</sub> was checked using two solvent systems for ITLC (A: 10 mM DTPA pH 4 and B: ammonium acetate 10% : methanol (1 : 1)).

*Radiolabeling of zoledronate with <sup>166</sup>HoCl<sub>3</sub>*

In order to obtain maximum complexation yield, several experiments were carried out by varying different reaction parameters such as ligand concentration, pH, and reaction time. The radiochemical purity of more than 99% was achieved at pH 7-8 and 40-50 °C with 40:1 ligand molar ratio to calculated holmium content. The reaction mixture was incubated at room

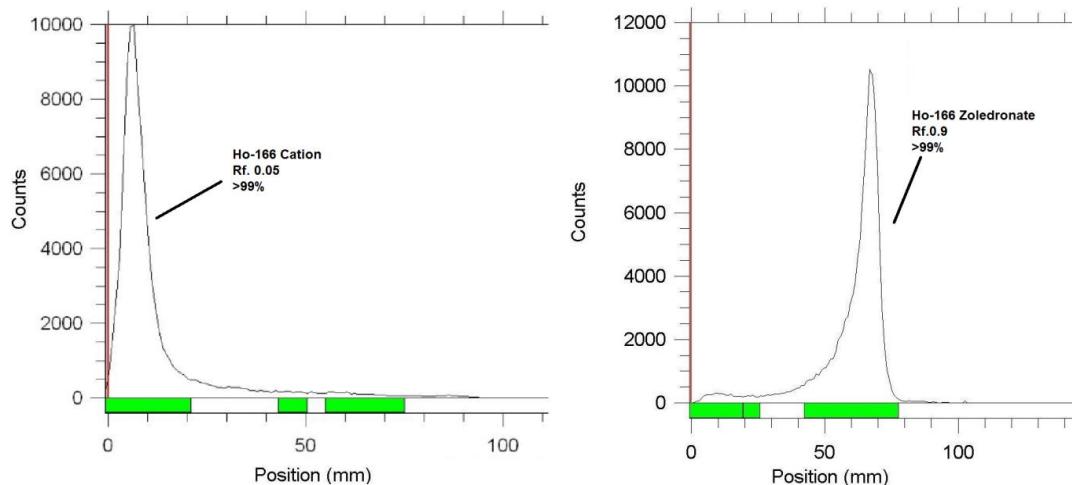
temperature for different time periods and 120 min incubation was found to be adequate to yield maximum complexation. The best ITLC mobile phase was considered Whatman 3 MM paper using NH<sub>4</sub>OH: MeOH: H<sub>2</sub>O (0.2:2:4). In the chromatography system of choice, free holmium cation elutes to the R<sub>f</sub> of 0.05 while the radiolabelled bis-phosphonate complex migrates to higher R<sub>f</sub>s, i.e. 0.9 (Fig. 2)

#### *Stability studies*

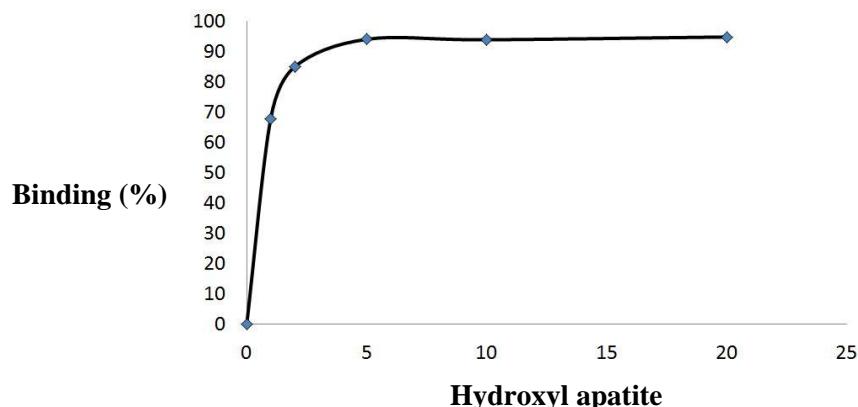
The stability of the complex prepared under optimized reaction conditions was studied at room and in presence of human serum at 37 °C. It was observed that the complex was > 95% stable both at room temperature and in presence of human serum at 37 °C even after 48 h.

#### *Hydroxy apatite assay*

The assay demonstrated high capacity binding for <sup>166</sup>Ho-Zoledronate to hydroxy apatite. Even at 2-3 mg amount of HA, more than 85% binding was observed, while at 5 mg HA used > 92% of binding was obtained. Compared to the other radiolanthanide zoledronate complex, i.e., <sup>153</sup>Sm-zoledronate, the holmium complex demonstrates higher hydroxy apatite binding, while Sm complex reaches the 90% affinity at 15 mg of hydroxyapatite (16), <sup>166</sup>Ho-zoledronate, retains this binding at 2-3 mg which is far better *in vitro* data for a bone seeking bis-phosphonate (Fig. 3).



**Figure 2** ITLC chromatograms of <sup>166</sup>HoCl<sub>3</sub> (left) and <sup>166</sup>Ho-Zoledronate solution (right) using Whatman 3 MM eluted with NH<sub>4</sub>OH: MeOH: H<sub>2</sub>O (0.2:2:4)



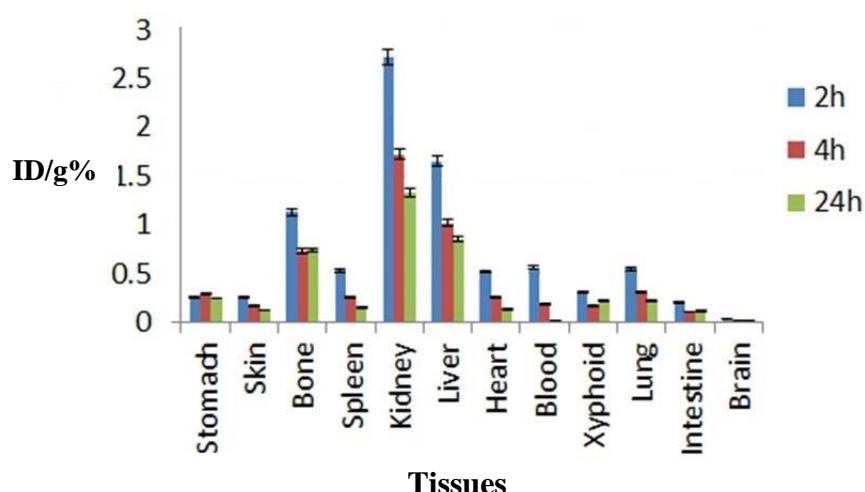
**Figure 3**  $^{166}\text{Ho}$ -Zoledronate binding assay to hydroxyapatite (1-20 mg)

#### Biodistribution of $^{166}\text{Ho}$ cation and $^{166}\text{Ho}$ -zoledronate in wild-type rats

The animals were sacrificed by CO<sub>2</sub> asphyxiation at selected time points after tracer injection. Dissection began by drawing blood from the aorta followed by removing the heart, spleen, muscle, bone, kidneys, liver, intestine, stomach and lungs samples. The tissue uptakes were calculated as the percent of area under the curve of the related photo peak per gram of tissue (%ID/g). For  $^{166}\text{Ho}^{3+}$  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 (Fig. 4).

#### Discussion

Radiolabelling of the complex using the carrier added Ho-166 produced in the research reactor was consistent with other related radiolanthanides as reported previously (4-7). Variations in complexation yields with respect to the zoledronate:holmium molar ratio as shown in Table 1 led to optimization of ligand concentration used. At pH 7-8 and 40 °C the best saturated molar ratio was observed for 50:1 ratio, while a major drop in radiochemical purity is observed in reducing 20 to 10. The stability of the complex at least in vitro was encouraging to initiate the animal studies according to the ethical animal codes.



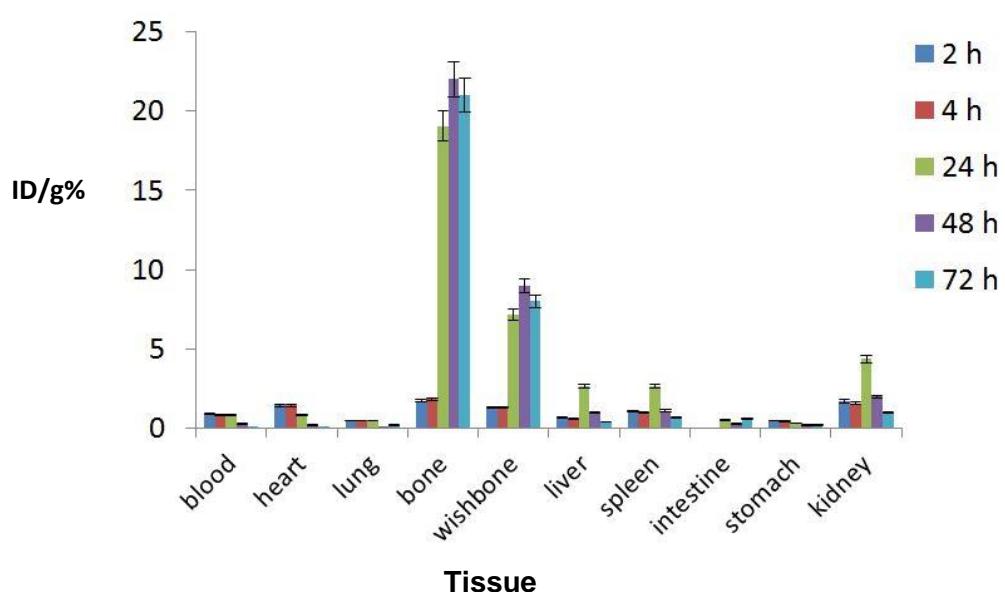
**Figure 4** Percentage of injected dose per gram of  $^{166}\text{HoCl}_3$  in wild-type rat tissue after 2, 4, and 24 h post injection

**Table 1** Complexation yields of <sup>166</sup>Ho-Zoledronate at different [ligand]:[metal] ratios

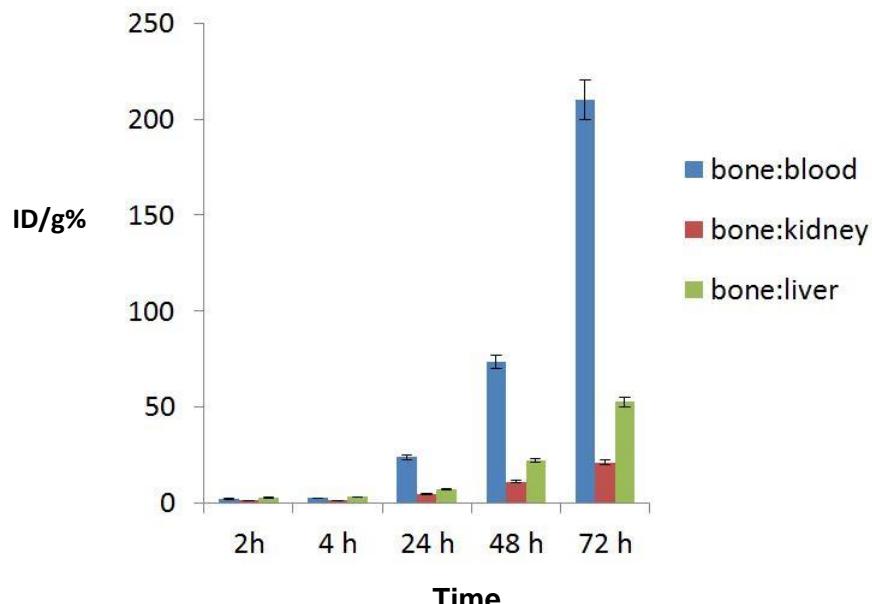
[ligand]:[metal] ratios	Radiochemical purity (%)
1:1	4
1:10	24
1:20	43
1:30	67
1:50	99
1:75	99

On the other hand, hydroxy apatite binding was also higher than 95% which is in accordance with in vitro data obtained for other bisphosphonate complexes (4-7).

Approximately 1.5% of the <sup>166</sup>Ho cation accumulates in the liver after 2 h which is comparable with many other radio-lanthanides mimicking calcium cation accumulation. Ho-166 activity is washed out as a water soluble free cation through kidney in 24 h. Ho-166 cation is present in other tissues are negligible. The distribution of injected dose in wild-type rat organs up to 72 h after injection of <sup>166</sup>Ho-zoledronate was also determined. As it can be seen in Figure 5, the major portion of the injected activity of <sup>166</sup>Ho-zoledronate was accumulated in bones as expected for bone-avid radiopharmaceuticals. For better understanding the ratios of target:non target for this complex in various time intervals Figure 6 demonstrates the increasing the uptake



**Figure 5** Percentage of injected dose per gram of <sup>166</sup>Ho-zoledronate in wild-type rat tissue after 2, 4, 24, and 48h post injection



**Figure 6** Comparative Bone:Blood, Bone:liver, Bone:Kidney activity ratios for  $^{166}\text{Ho}$ -zoledronate in 2-72 h

ratio with increasing time up to 72 h. Compared to other reported Ho-166 labeled bisphosphonates such as  $^{166}\text{Ho}$ -DOTMP (18), the bio distribution of this complex looks superior, however the slightly higher liver uptake in this case is a drawback. On the other hand non-cyclic bisphosphonate complexes such as  $^{166}\text{Ho}$ -pamidronate have shown far worse biodistribution data leading to higher kidney and liver uptake possibly due to in vivo stability (19). The only non-cyclic bis phosphonate showing good performance in animal data has been  $^{166}\text{Ho}$ -EDTMP (20) with almost acceptable stability and bone targeting. The aim of radionuclide therapy is complete ablation of the target with minimal radiation exposure to other organs. Target to non-target ratio is the most important parameter in the selection of radiopharmaceuticals for therapy of choice for each malignancy. Since  $^{166}\text{Ho}$ -zoledronate was produced as a bone marrow ablative agent, red marrow to other tissue dose must be determined.

### Conclusion

$^{166}\text{Ho}$ -zoledronate complex was prepared in high radiochemical purity (>99 %, ITLC) and specific activity of 4.4 GBq/mmol. The complex demonstrated significant stability at room temperature and in presence of human serum at 37 °C even after 48 h. The final preparation was administered to wild-type rats and biodistribution of the complex was checked 2-72 h post injection showing major accumulation of the complex in the bone tissue. The highest absorbed dose for  $^{166}\text{Ho}$ -zoledronate is observed in bone tissue. Compared to the other radiolanthanide zoledronate complexes,  $^{166}\text{Ho}$ -zoledronate demonstrates higher hydroxy apatite binding and also better bone:liver ratio. According to the results,  $^{166}\text{Ho}$ -zoledronate has considerable characteristics and can be a good candidate for bone marrow ablation in patients with multiple myeloma.

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