Original Article

Radiosynthesis and Animal Studies of ^{111}In-DTPA-*bis*-alendronate Complex

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ABSTRACT

Purpose- In order to develop new bone imaging agent based on bisphosphonate moiety, an alendronate based complex was designed and prepared in this study.

Methods- A DTPA-conjugated bis-alendronate analog (DTPA-*bis*-ALN) 3, was prepared for possible bone imaging after radiolabeling with ^{111}In. Radiolabeled DTPA-*bis*-ALN complex was prepared starting radionuclide in chloride form and DTPA-*bis*-ALN in 30-90 min at 50-60°C in acetate buffer followed by solid phase purification on C_{18} Sep-Pak cartridge. RTLC was used for radiochemical purity determination followed by log P determination, stability studies, hydroxyl apatite tests and biodistribution studies in normal rats and imaging.

Results- Specific activity 1.1-1.3 TBq/mmmol was obtained for ^{111}In-complex. The log Ps was calculated 0.48 for the complex consistent with water soluble complexes followed by stability test. The biodistribution of the labeled compound in normal rats demonstrated activity uptake in kidneys.

Conclusions- The anionic property of poly-dentate complex led to renal excretion instead of bone accumulation. These data showed that the developed complexes are not suitable agents for bone imaging as expected.

1. Introduction

Due to significant inhibitory binding affinity constant (Ki) of bis-phosphonates used in clinics esp. alendronic acid, the idea of developing its bone avid agents is of great interest. In recent studies, using simple bis-phosphonate radiolanthanide complexes such as ^{177}Lu-zoledronate [1], ^{166}Ho-pamidronate [2], ^{177}Lu-pamidronate; ^{166}Ho-alendronate [3] and ^{177}Lu-alendronate [4] have shown significant bone uptake however no superior bone avidity compared to their clinical rivals. The resulting complexes were not stable in vivo and/or showed lower bone uptake compared to other therapeutic bis-phosphonates.

Thus another interesting approach was to develop bis-phosphonate ligands including metal chelating agents including DTPA moiety as already reported for Tc-99m analog [5]. In this work, a multi dentate poly-amino carboxylic acid containing bis-phosphonate ligand, presumably to form stable chelates with many metals including lanthanides, was developed by conjugation of cyclic DTPA dianhydride and [4-amino-1-hydroxy-1-(hydroxy-oxido-phosphoryl)-butyl] phosphonic acid (Alendronic acid), as a possible carrier moiety.

The interesting physical properties of ^{111}In [a cyclotron produced radionuclide with half-life of 2.8 d, decaying by electron capture (EC) with subsequent emission of gamma photons of 173 and

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247 keV (89% and 94% intensity, respectively)] as well as its easily production and availability, make it an attractive nuclide for radiopharmaceutical research. Various carriers including peptides and antibodies labeled with ^{111}In have shown the usefulness of this radioisotope in the detection and diagnosis of cancers using single photon emission computed tomography (SPECT). Recently the development of ^{111}In-BPAMD as a bone-avid agent has been reported [6]. In continuation of those works, we report synthesis, purification and preclinical evaluation of ^{111}In-DTPA-*bis*-ALN for imaging purposes.

2. Materials and Methods

2.1. Experimental

The production of ^{111}In was performed at the Radiation Application Research School, Karaj, Iran, using the ^{112}Cd(p,2n)^{111}In reaction. Whatman No. 2 was obtained from Whatman (Maidstone, UK). Radio-chromatography was performed by using a Bioscan AR-2000 radio TLC scanner instrument (Bioscan, Paris, France). 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 rats organs. All other chemical reagents were purchased from Merck (Darmstadt, Germany). Calculations were based on the 172 keV peak for ^{111}In. The approval of NSTRI Ethics Committee was obtained for conducting this research. The wild-type rats (NMRI) were purchased from Pasteur Institute of Iran, Karaj, and all weighing 120-150 g and were acclimatized at a proper rodent diet and 12 h/12 h day/night light/darkness. Scintigraphic images were obtained using a dual-head SPECT system.

2.2. Production and Quality Control of $^{111}InCl_3$ Solution

Cadmium electroplating over a copper surface, the production of ^{111}In in chloride form as well as quality control were performed according to reported method by 22 MeV proton bombardment of the enriched cadmium-112 target at the 30 MeV cyclotron (from IBA), with a current of 100 μA for 48 min (80 μAh) [7].

2.3. Preparation of Fresh Cyclic DTPA Dianhydride

Cyclic DTPA dianhydride was prepared according to the reported method with slight modifications [8]. Briefly, DTPA in acidic form (0.1 mole) was heated with a 4-fold molar excess of acetic anhydride (0.4 mole), dissolved in 50 mL of pyridine and heated at 65°C for 24 h. The resulting anhydride was insoluble in pyridine and was collected by filtration, purified by repeated washing with acetic anhydride, and finally with anhydrous ether. Drying in an oven at 50-60°C removed the last traces of solvent. The melting point of white solid was 182-185 °C and its ^{1}H NMR and IR spectra were in accordance with the literature.

2.4. Conjugation of Cyclic DTPA Dianhydride with Alendronate

The DTPA conjugation to alendronate was performed based on reported similar method with slight modifications [9]. To a magnetically stirred solution of cyclic DTPA dianhydride (108 mg, 0.3 mmol) in anhydrous DMF (2 mL) was added triethylamine (0.16 mL, 1.2 mmol) under nitrogen (final pH. 8). After 15 min a solution of alendronic acid (32.7 mg, 0.08 mmol) in DMF (1 mL) was added drop wise to the reaction mixture at room temperature for 30 min. The reaction mixture was then allowed to stir for 24 h at 60ºC. A few drops of water were added to quench the reaction. Diethyl ether was added to the reaction mixture and the precipitate was recovered and further purified using flash chromatography. Yield: 48% (56 mg). The structure of compound was determined using spectroscopic methods. ^{1}HNMR (500 MHz, D_2O), 7.72 (bs, 2H, -CO-NH-), 3.61 (s, 8H, -CH_2-COOH), 3.26 (t, 4H, -CH_2-NH_2), 3.22 (s, 2H, CH_2-COOH), 2.8 (s, 8H, N-CH_2-CH_2-N), 2.6 (s, -OH), 1.8 (t, 8H, -CH_2-CH_2-). MS: m/z cal. 889, found [M+2H_2O] + 935 IR (KBr):1552, 1648, 3307 cm^{-1},

2.5. Radiolabeling of DTPA-*bis*-ALN with Radionuclides

A stock solution of DTPA-*bis*-ALN in pure

ethanol was prepared (20 mg.mL^{-1}). For labeling, an appropriate amount of the radionuclide solution containing the required activity (0.1 mL, 4 mCi) was added to the desired amount of DTPA-*bis*-ALN solution (60 mL). The pH adjusted using acetate buffer to 5. The complex solutions were kept at room temperature for 1-6 h. Also another set of experiment was performed at 60ºC warm bath for 1-2 h. The 80-88% radiochemical purity was obtained using paper chromatography. For further purification the reaction mixtures were passed through a freshly activated C_{18} Sep-Pak cartridge (pre-washed by ethanol and water 4 and 2 mL respectively) and fractions were collected by washing with water:methanol (1:1 v/v) mixture. The final solution was passed through a 0.22 µm membrane filter and pH was adjusted to 7-8.5 with 0.05 molL^{-1} acetate buffer (pH 5.5). For Radiochemical purity of the complexes, an instant thin layer chromatography was used. A 5 ml sample of the final fraction was spotted on a chromatography Whatman No. 2, paper, and developed in Whatman 2 MM chromatography paper eluted with NH_4OH (56%): MeOH (%100): H_2O (%100) (0.2:2:4; v/v/v) as mobile phase mixture.

2.6. Stability of the Radiolabeled Complexes in Final Formulation and Serum

For serum stability studies, 300 mL of freshly prepared healthy human serum was added to 7.4MBq (200 mCi, 100 mL) of radiolabeled complex final solution and the resulting mixture was incubated at 37ºC for 48 h. Every 12 h to a portion of the 50 mL of the mixture, trichloroacetic acid (10%, 100 mL) was added and the mixture was centrifuged at 3000 rpm for 5 min followed by decanting the supernatant from the debris. The stability was determined by paper chromatography analysis of supernatant using Whatman 2 MM chromatography paper eluted with NH_4OH (56%): MeOH (%100): H_2O (%100) (0.2:2:4; v/v/v).

2.7. Hydroxyapatite Binding Assay

The hydroxyapatite binding assay was performed according to the procedure described previously [10], 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, 2 mL of saline solution of pH 7.4 were added and the mixtures were shaken for 1 h. Then, 50 µL of the radioactive preparation 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 radiolanthanide 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.

2.8. Biodistribution Studies

The biodistribution of free cations as well as of the radiolabeled complexes were determined in normal rats. For each compound, 100 µL (150 µCi) of radioactive solution was injected directly to normal rats through caudal vein. The animals (n=5) were sacrificed at selected times after injection and the percentage of injected dose in the tissues was determined with a g-ray scintillation or a dose calibrator.

2.9. Imaging of [^{111}In]-DTPA-*bis*-ALN in Rats

Rats injected with [^{111}In]-DTPA-*bis*-ALN were used for imaging. Images were taken at various time intervals after administration of the tracer by a dual-head SPECT system (SMV, France, Sopha DST-XL). The rat-to-high energy septa distance was 12 cm. The useful field of view (UFOV) was 540 mm×400 mm. The spatial resolution was 10 mm FWHM at the CFOV. Sixty four projections were acquired for 30 seconds per view with a 64×64 matrix.

3. Result

Synthesized DTPA cyclic anhydride was characterized by IR spectroscopy. The formation of 1730 cm^{-1} peak indicated an anhydride carbonyl group formation which is accompanied by a weaker 1695 cm^{-1} carboxylic acid peak of

the untouched COOH. ^{1}H NMR spectrum of the above compound was recorded in DMSO at 25°C. The chemical shifts of CH_2CO groups have the lowest field are very close so that a major singlet is observed around 3.76 ppm. The NCH_2CH_2N groups are more shielded and because of their similarity, a broad multiplet is observed at 2.6-2.56 ppm. The DMSO peak is observed at 2.5 ppm as a multiplet [11].

Various reaction conditions were tried for DTPA-*bis*-ALN conjugation. The conjugation was performed in DMF, DMSO as well as aqueous mixtures as the best reaction solvent showed to be DMF since the reaction work-up seemed feasible due to the precipitation of the final compound followed by solid washing using various solvents. For better yields the alendronate free base (1) was added to the ccDTPA (2) and not vice versa.

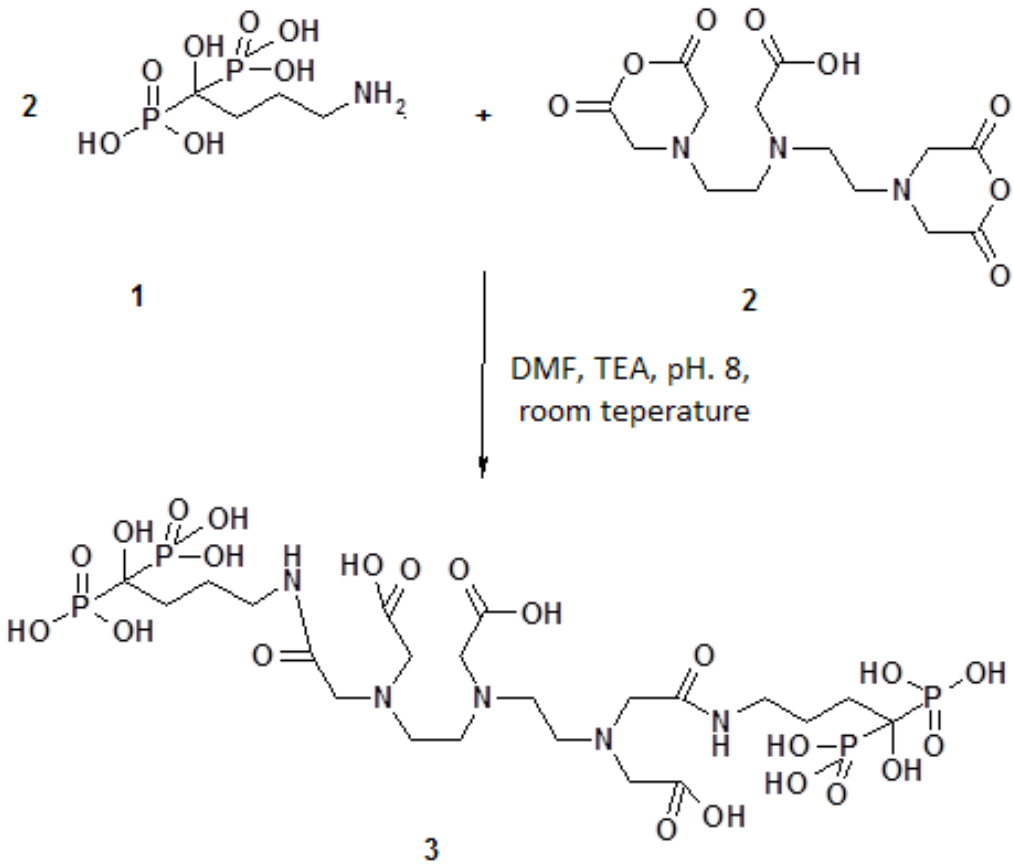

Figure 1. Reaction steps for preparation of DTPA-*bis*-ALN conjugate.

Nitrogen atmosphere was also mandatory for the conjugation reaction since the presence of water can reduce the conjugation yields. The reaction scheme is shown in Figure 1.

3.1. Radionuclide Production

Indium-111, in form of $InCl_3$, was prepared by 22 MeV proton bombardment of the enriched ^{112}Cd target at Cyclone-30 on a regular basis. Radionuclidic control showed the presence of 171 and 245 keV gamma energies, all originating from ^{111}In and showed a radionuclidic purity higher than 99% (E.O.S.). The concentrations of cadmium (from target material) and copper (from target support) were determined using polarography and shown to be below the internationally accepted levels, i.e. 0.1 ppm for Cd and Cu [12,13]. 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.

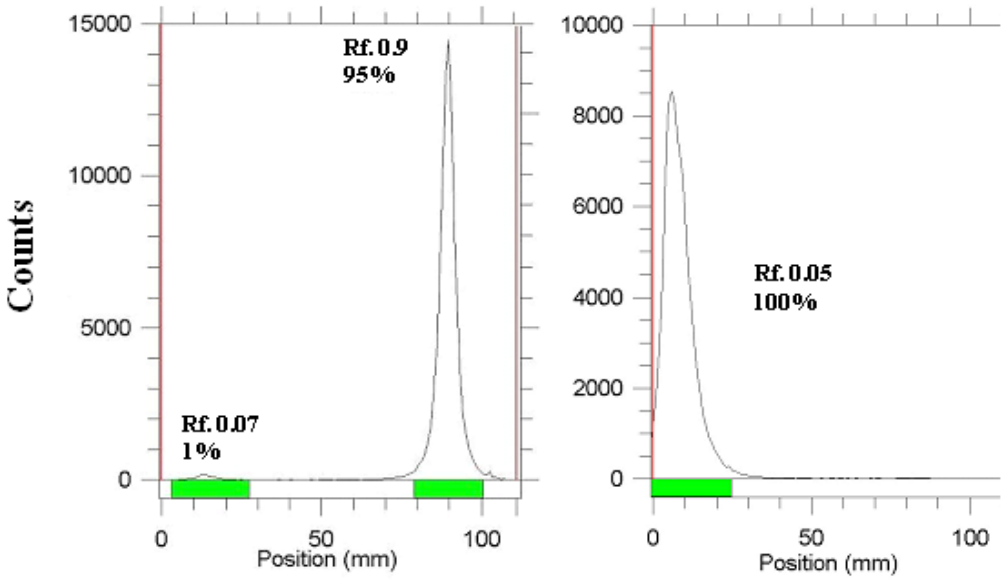

Figure 2. Chromatograms of ^{111}In cationic solutions in 10 mM DTPA solution (pH~ 4) (left) and in 10% ammonium acetate: methanol (1:1) (right) on Whatman No. 1 Paper.

3.2. Radiolabeling

The radiochemical purity of the radionuclide solution was checked in two solvent systems, in 1mM DTPA, free In^{3+} cations is converted to more lipophilic metal-DTPA form and migrates to higher R_f (0.8-0.9) while any small radioactive fractions remaining at the origin could be related to other metallic ionic species, not forming In-DTPA complex, such as $InCl_4^-$, etc. and/or colloids (not observed).

On the other hand, 10 % ammonium acetate: methanol mixture was also used to determine the radiochemical purity. The fast eluting species was possibly the metal cations other than In^{3+} (not observed) and the remaining fraction at R_f=0 was a possible mixture of In^{3+} and/or colloids (Figure 2).

Because of the engagement of NH and OH polar functional groups in DTPA-*bis*-ALN structure, labeling of DTPA-*bis*-ALN with metal cations affects its chromatographic properties and the final complex is more lipophilic. Chromatographic system was used for the detection of the radiolabeled compound from the free cation (Figure 3).

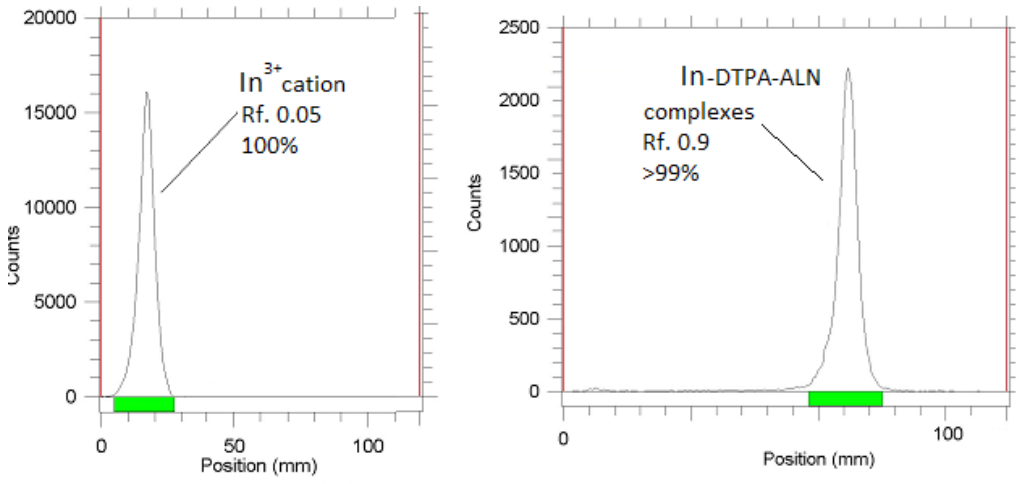

Figure 3. Chromatograms of $InCl_3$ (left) and In-DTPA-*bis*-ALN complex (right) using Whatman No. 2 Paper eluted with 10% ammonium acetate and MeOH 1:1 mixture.

3.3. Labeling Studies

Using 10% ammonium acetate and MeOH 1:1 mixture, cation remains at the origin of the paper as a single peak, while the radiolabeled compound migrates to higher R_f (0.8) (Figure 4). In order to obtain maximum complexation yields, several experiments were carried out varying different reaction parameters such as ligand concentration, pH, reaction time and temperature. Ligand concentration was varied between a wide range starting from 10 to 50 mg/mL for DTPA-*bis*-ALN. It was observed that at room temperature 99% complexation was achieved with 15 mg/ml of DTPA-*bis*-ALN. The best paper chromatography mobile phase was considered by Whatman No.2 paper using NH_4OH: MeOH: H_2O (0.2:2:4) as shown in Figure 4.

In order to achieve higher specific activities for the final labeled compounds, excess amounts of radionuclide were used in the radiolabeling procedure; however a solid phase purification step using C_{18} Sep-Pak was applied to the final mixtures in order to get higher radiochemical purities by removing excess amounts of unlabeled cations. By fraction eluting of the loaded cartridges applying the same solvent system used in the radio chromatography of the labeling procedure, the radiolabeled complex was eluted in the first 1-3 mL fractions. Figure 4 demonstrates the elution of the unlabeled cations from the column.

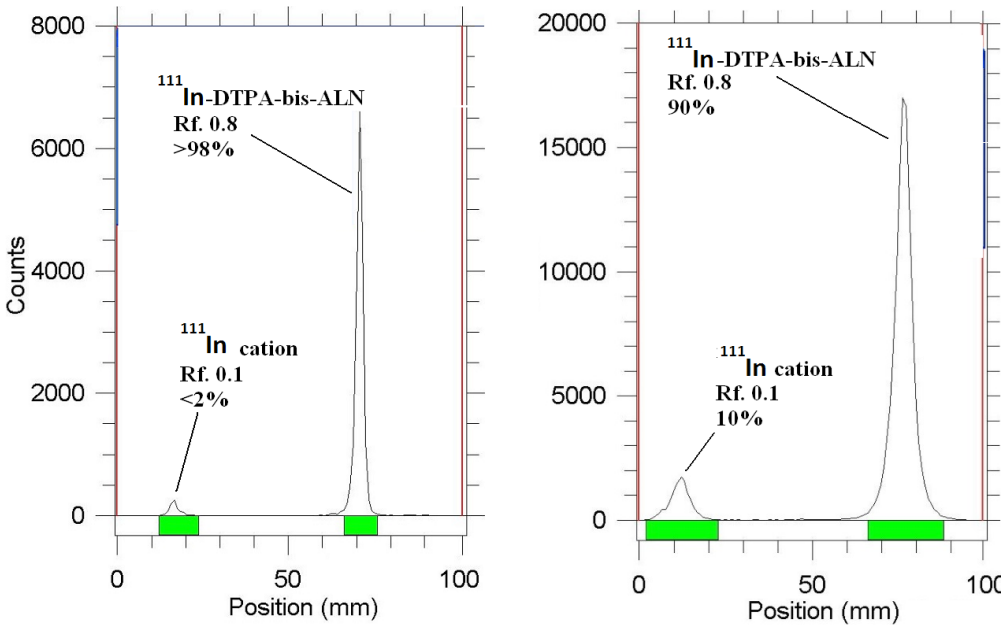

Figure 4. Chromatograms of ^{111}In-DTPA-*bis*-ALN solutions prior to solid phase purification (right) and after solid phase purification (left) in NH_4OH: MeOH: H_2O (0.2:2:4) as mobile phase on Whatman No. 2 Paper.

The stability of prepared complex was checked up to 24 hours after preparation. The complexes were stable in the final pharmaceutical samples and their radiochemical purity were the same as the beginning even 24 hours after preparation using Whatman 2 MM eluted with NH_4OH: MeOH: H_2O (0.2:2:4).

Stability test was developed for the complex in presence of human serum at 37ºC using paper chromatography as mentioned above and all data within 48 were above >98% at all-time intervals.

HA assay demonstrated low capacity binding for all DTPA-*bis*-ALN complexes to hydroxy apatite. Even at 50 mg amount of HA, less than 29-31% binding was observed (Table 1).

Table 1. Hydroxy apatite binding assay for complexes at 37ºC in 24 h, n=5.

Ligand / hydroxyapatite	5 mg	10 mg	15 mg	20 mg	25 mg	50 mg
^{111}In-DTPA-*bis*–ALN	15±0.23%	18±0.3%	20±0.93%	25±0.32%	30±0.32%	31±0.53%

3.4. Biodistribution

For better comparison biodistribution study was performed for free cation as well. The %ID/g data for free indium-111 cation is summarized in Figure 5.

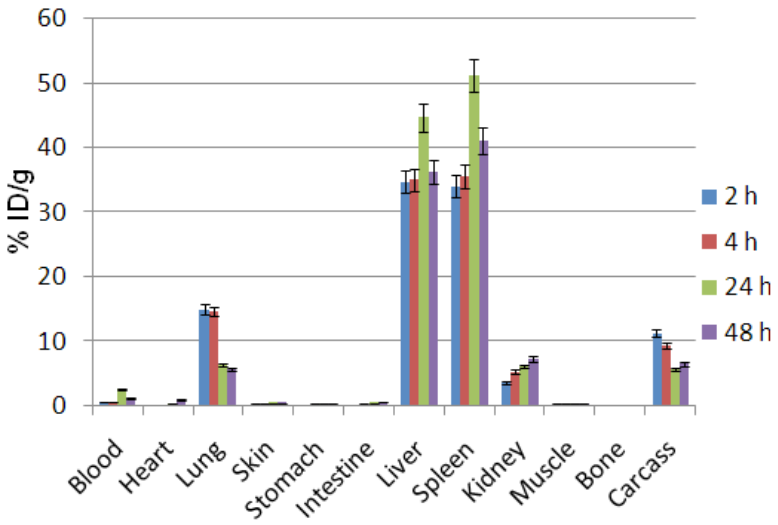

Figure 5. Biodistribution of $^{111}InCl_3$ (1.85 MBq, 50mCi) in normal rats at various time intervals after iv injection in 2-48 h post injection via tail vein (ID/g%: percentage of injected dose per gram of tissue) (n=5).

As reported previously, indium cation almost mimics the ferric cation behavior and is rapidly removed from the circulation and is accumulated in the liver, about 10-15 % of the activity accumulates in the liver after 24 h also a major fraction is excreted through the urine as a water soluble cation. This behavior has been formerly shown in 1980 s in human studies and experimental animals [14].

3.5. Biodistribution of Radiolabeled DTPA-*bis*-ALN Complexes

In case of ^{111}In -complex also the high solubility of the complex is observed leading to high tracer uptake in kidney at all-time intervals and almost all the activity is excreted through the urinary tract without significant uptake in liver lung etc. (Figure 6).

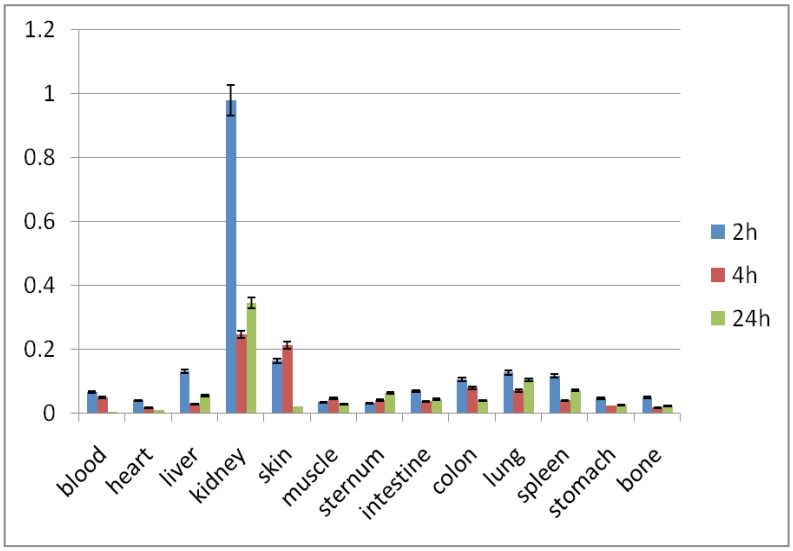

Figure 6. Biodistribution of [^{111}In]-DTPA-*bis*-ALN (1.85 MBq, 50 mCi) in wild-type rats 2, 4 and 24 h after iv injection via tail vein (ID/g%: percentage of injected dose per gram of tissue calculated based on the area under curve of 172 keV peak in gamma spectrum) (n=5).

3.6. Imaging Studies of [^{111}In]-DTPA-*bis*-ALN

Using imaging studies, the biodistribution of ^{111}In-radioconjugate was observed in in 2-24 h time interval; which was in full accordance with biodistribution results. The high activity uptake in 2 h is observed in bladder and also kidney. The kidneys uptake remained constant possibly due to cellular kidney affinity of the tracer as observed for some former tracers with negative charge (Figure 7).

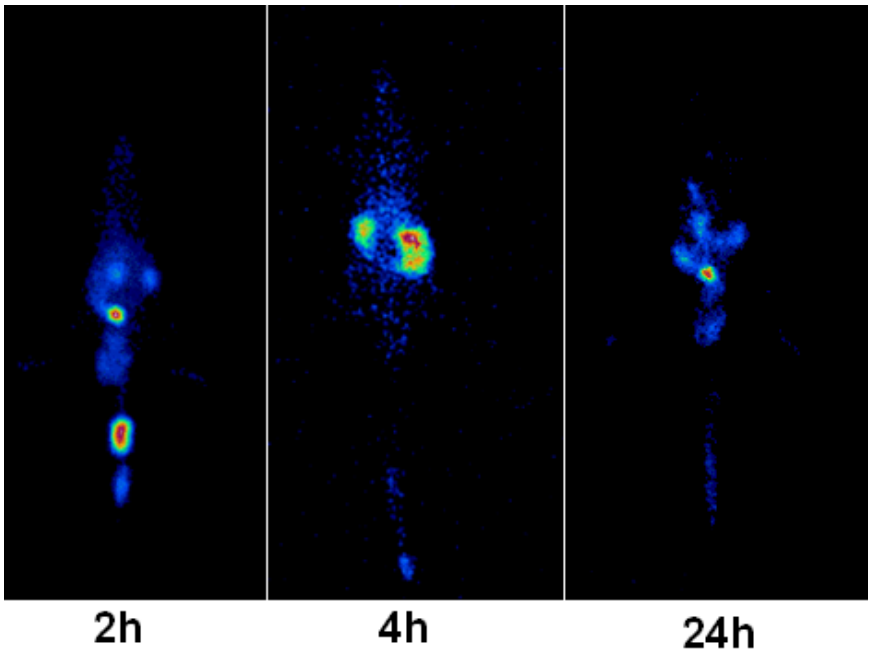

Figure 7. Imaging of normal rats 2-24 h post injection of [^{111}In]-DTPA-*bis*-ALN (3.7-4 MBq, 100-110 mCi).

4. Discussion

Radiolabeled DTPA-*bis*-ALN complex was prepared with significant specific activity and negative log Ps consistent with water soluble complex followed by acceptable stability. Hydroxyl apatite binding (towards 5 mg) was low for the complex. The biodistribution of the labeled compound in normal rats demonstrated unwanted activity uptake in kidneys. Very limited bone uptake in all cases demonstrating complex instability or the loss of bone avidity due to change of structure-activity relationship and/or anionic property of poly-dentate complex leading to renal excretion. These data showed that the developed complexes are not suitable agents for bone imaging as expected. The development of an alendronate based bone seeking agent possibly must be directed to a 1:1 alendronate: DTPA moiety with lower negative charges on the molecule and more stereo-availability for hydroxyl apatite molecules on the bone tissue. These data showed that the developed complexes are not suitable agents for bone imaging as expected.

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References

1- M. Nikzad, A. R. Jalilian, S. Shirvani-Arani, A. Bahrami-Samani, H. Golchoubian, "Production, quality control and pharmacokinetic studies of 177Lu-zoledronate for bone pain palliation therapy," *J Radioanal Nucl Chem,* vol. 298 pp. 1273–1281, 2013.

2- A. Fakhari, A. R. Jalilian, H. Yousefnia, F. Johari-Daha, M. Mazidi, A. Khalaj, "Development of ^{166}Ho-pamidronate for bone pain palliation therapy," *J Radioanal Nucl Chem,* vol. 303, pp. 743–750, 2015.

3- A. Fakhari, A. R. Jalilian, H. Yousefnia, A. Bahrami

Samani, M. R. Akbari, F. Johari-Daha, M. Sharifi-Ardestani, A. Khalaj, "Production, quality control, biodistribution assessment and preliminary dose evaluation of ^{166}Ho-alendronate as a bone marrow ablative agent," *Radiochimica Acta,* in press, 2015.

4- A. Fakhari, A. R. Jalilian, H. Yousefnia, A. Bahrami-Samani, F. Johari-Daha, A. Khalaj, "Evaluation of two ^{177}Lu-labeled *bis*-phosphonates for bone pain palliation therapy," *Iran J Nucl Med,* vol. 23, pp.108-115, 2015.

5- N. Chadha, D. Sinha, A. K. Tiwari, K. Chuttani, A. K. Mishra, "Synthesis, Biological Evaluation and Molecular Docking Studies of High-Affinity Bone Targeting N,N'-Bis (alendronate) Diethylenetriamene-N,N'-Triacetic Acid: A Bifunctional Bone Scintigraphy Agent," *Chem Biol Drug Des,* vol. 82, pp. 468–476, 2013.

6- H. Yousefnia, A. R. Jalilian, et al., "Preparation and biodistribuion assessment of ^{111}In-BPAMD as a novel agent for bone SPECT imaging," *Radiochimica Acta,* in press, 2015.

7- A. Khorami-Moghadam, A. R. Jalilian, K. Yavari, B. Alirezapour, M. Mazidi, M. Mirzaii, "Synthesis, radiolabeling and quality control of ^{111}In-DOTA-bevacizumab for radioimmunoscintigraphy of VEGF receptors," *Radiochimica Acta,* vol. 101, pp.577-584, 2013.

8- D. J. Hnatowich, W.W. Layne, R. L. Child, "Radioactive labeling of antibody: A simple and efficient method," *Science,* vol. 220, pp. 613-619, 1983.

9- A. Fakhari, A. R. Jalilian, H. Yousefnia, S. Shanesazzadeh, A. Bahrami-Samani, F. Johari-Daha, M. Shafiee-Ardestani, A. Khalaj, "Preparation, biological evaluation and dosimetry studies of ^{175}Yb-bis-phosphonates for bone pain palliation therapy," *Molecular Imaging and Radionuclide Therapy,* in press, 2015.

10- M Neves, L Gano, N Pereira, MC Costa, MR Costa, M Chandia, *et al.,* "Synthesis, characterization and biodistribution of bispho- sphonates Sm-153 complexes: correlation with molecular modeling interaction studies," *J Nucl Med Biol,* vol. 29, pp. 329–338, 2002.

11- T. Firouzyar, A. R. Jalilian, M. Shafiee-Ardestani, M. R. Aboudzadeh, Y. Fazaeli, F. Bolourinovin, M. Mirzaii, A. Khalaj. "Development of an ^{111}In-labeled dihydropyridine complex for L-type calcium channel imaging," *J Radioanal Nucl Chem,* vol. 303, pp. 2361–2369.

12- United States Pharmacopoeia 28, NF 23, p.1009 (2005).

13- United States Pharmacopoeia 28, NF 23, p.1895 (2005).

14- Bettye A. Sayle, Suppiah Balachandran, and Charles A. Rogers. "Indium-111 Chloride Imaging in Patients with Suspected Abscesses: Concise Communication," *J Nucl Med,* vol. 24, pp. 1114-1118, 1983.