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ORIGINAL RESEARCH ARTICLE

An optimized formulation for [^{99m}Tc]Tc radiolabeling of zoledronic acid as bone imaging agent

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ABSTRACT

Introduction: The aim of this study was to develop an optimized formulation for labeling a third-generation bisphosphonate, zoledronic acid with [^{99m}Tc] Tc to achieve the best formulation in preparing an ideal skeletal radiotracer. Radio-complex yield and purity, stability, biodistribution and imaging in normal rat were investigated.

Methods: The samples containing different amounts of zoledronic acid, ascorbic acid and stannous chloride were prepared and labeled with [^{99m}Tc]technetium pertechnetate. TLC methods were used to determine the radiochemical purity. The stability was determined in saline and human serum solutions. Lipophilicity was calculated by measuring radio-complex that was divided between organic and aqueous phases. In vitro bone affinity was studied through hydroxyapatite binding assays. Considering the decomposition of radioactivity, biodistribution of radio-complex was assessed based on the percentage of injected activity per gram of organ (% IA/g).

Results: [^{99m}Tc]Tc-zoledronic acid was prepared easily with high yield while 100 µg, 0.34 µmol of zoledronic acid as a ligand and 100 µg, 0.44 µmol $SnCl_2$ as a reducing agent were used. Radiochemical purity of radio-complex was more than 99% with specific activity of 8050 MBq/µmol. The radio-complex showed rapid blood washout along with high bone uptake value (4.53 ± 0.14 % IA/g at 2 h post injection).

Conclusion: Under optimized condition, [^{99m}Tc]Tc-zoledronic acid was prepared with high purity and stability together with high bone affinity and rapid blood clearance, make this radio-complex an ideal agent with great potential for skeletal imaging.

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INTRODUCTION

Bisphosphonates are synthetic analogues of pyrophosphate distinguished by a P-C-P backbone that makes them resistant to hydrolysis. The carbon side chains determine the pharmacological properties of the bisphosphonates. These side chains are able to chelate divalent metal ions by coordinating one oxygen from each phosphonate group with the divalent cation. When bisphosphonates contain a hydroxyl group at one side chain of carbon, this binding could enhance and thus enable tridentate interaction [1]. In bisphosphonates structure a non nitrogen-containing or nitrogen-containing side chain on the other side of carbon, is responsible for antiresorptive potency [2, 3]. The earlier generation of these compounds like etidronate include short side chain. Second generation compounds have aliphatic chains of different lengths bearing terminal amino groups like alendronate or substituted amino groups like olpadronate [4]. The third-generation bisphosphonates like zolendronic acid with heterocyclic nitrogen containing side chain have a high affinity for hydroxyapatite crystals in bone and are strong antiresorptive agents [5]. At the cellular level, risedronic acid inhibits osteoclasts, which results in reducing bone turnover [6].

While for more than thirty years [^{99m}Tc]Tc labeled bisphosphonates especially [^{99m}Tc]Tc-MDP and [^{99m}Tc]Tc-HMDP are widely used in clinics as an ideal bone imaging agents, but there are some disadvantages about them. As these compounds are a mixture of short and long chain oligomers and cannot exist as a single species, their exact structures are still unknown. As well as the biological properties of the compound can be changed by the relative number of oligomers, the different degrees of ionization and in vivo degradation after preparation [7]. Furthermore it is known that the tracer do bind to Ca^{2+} of hydroxyapatite crystals [8], so the phosphonate groups in [^{99m}Tc]Tc-bisphosphonate complexes such as [^{99m}Tc]Tc-MDP and [^{99m}Tc]Tc-HMDP serve as both 5 coordinating ligand and Ca^{2+} binding functional groups [9], which might decrease the intrinsic accumulation of tracers in bone. Moreover, from the standpoint of clinical studies, since [^{99m}Tc]Tc-bisphosphonates show slow blood clearance a delay time of 2-6 h is required to start the bone scanning [10]. Subtracting the time delay can reduce the trouble of the patient in connection with the total test length and radiation dose absorbed.

To overcome the above-mentioned drawbacks, preparing of a radio-complex with ideal characteristics such as higher absorption for bone, more rapid clearance from blood followed by possibility to image at an earlier time after injection is required. In order to achieve a tracer with high bone resorption, a third generation bisphosphonate, zoledronic acid, was considered to be labeled with [^{99m}Tc]Tc. Labeling zoledronic acid and its derivatives with different radionuclides have been previously reported [11-16]. Asikogulo et al. reported unsuccessful labeling with [^{99m}Tc]Tc when they used low amount of zoledronic acid in labeling process [11]. In this study, labeling formulation was optimized for preparing [^{99m}Tc]Tc-zoledronic acid using low amount of zoledronic acid and obtaining radio-complex with high specific activity. The prepared radio-complex was further investigated in terms of radiochemical purity, stability, lipophilicity, biodistribution and imaging in rat.

METHODS

Zoledronic acid monohydrate and all other reagents were purchased from Sigma/Aldrich and used without further purification. [^{99m}Tc]technetium pertechnetate was eluted from a commercial [^{99m}Mo]Mo/[^{99m}Tc]Tc generator (Iran, Tehran, Pars Isotope Co) with saline solution (0.9% NaCl). Radioactivity was determined in a dose calibrator (Isomed, Germany). Quantitative gamma counting was performed using an EG&G / ORTEC Model 4001M Mini Bin & Power Supply NaI(Tl) counter. RTLC analysis was carried out through a Raytest-GITA scanner (Germany).

Radiolabeling

A 0.003 M solution of zoledronic acid was prepared by dissolving zoledronic acid in 0.01 M citrate buffer. Samples of different amounts of zoledronic acid (0.05-5 mg) and ascorbic acid (0.2-2 mg) were combined in a vial. From 0.02 M solution of $SnCl_2$, 2H2O in nitrogen-purged 0.1 M HCl, different amounts (0.05-1 mg) in company with 370-1480 MBq of sodium pertechnetate ([^{99m}Tc]TcO4$^-$) in saline were added to the samples. Final pH was adjusted (pH=1-7) by addition of 1 N HCl. The mixture was shaken vigorously for 30 second. The incubation was carried out in sealed container for 15 minutes at room temperature.

Quality control

The radiochemical purity of the radio-complex ([^{99m}Tc]Tc-zoledronic acid) was determined by

thin layer chromatography (TLC) method. About 5 µL radio-complex solution was applied at 1 cm from the bottom of paper stripes (whatman 1) and then stripes were developed in acetone and phosphonic acid (15%) as solvents. After complete development, the chromatographic paper stripes were analyzed through TLC scanner to determine the radiochemical purity. With the acetone as an eluting solvent, unlabeled [^{99m}Tc]Tc ([^{99m}Tc]TcO4$^-$) move to the top of the strip and conjugate bound [^{99m}Tc]Tc ([^{99m}Tc]Tc-zoledronic acid) remain at the base. In paper strip developed with phosphonic acid 15% as an eluting solvent, only colloidal impurity ([^{99m}Tc]TcO2.χ H2O) remains at the origin while [^{99mTc}]TcO4$^-$ and [^{99m}Tc]Tc-zoledronic acid both migrate with the solvent front.

Stability and lipophilicity

Stability of radio-complex was studied in normal saline and human serum at different time interval. 100 µL of radio-complex was added into 1 mL of normal saline and human serum separately. After incubation at 37 °C for different time point (up to 24 h) an aliquot (100 µL) of saline and serum solution were evaluated for stability. To precipitate the protein, 100 µL ethanol was added to the serum sample and after centrifugation (3000 g, 5 min at 4 ºC) the supernatant was analyzed by TLC.

To determine lipophilicity, 100 µL of the radio-complex was mixed with 1 mL of octanol and 1 mL of water. The solution was vigorously vortexed and then centrifuged for 5 min at 3500 × g. An aliquot (50 µL) of each phase were taken and their radioactivity were measured. The octanol-water partition coefficient (log Po/w), was calculated by dividing the organic phase radioactivity to that of the aqueous phase.

Hydroxyapatite binding

[^{99m}Tc]Tc-zoledronic acid binding to bone matrix was performed through the following protocol. hydroxyapatite particles were suspended in normal saline (39 mM) and kept on stirring at room temperature for 24 h. 1 mL of suspension was added to a glass vial and 50 µL of the solution of [^{99m}Tc]Tc labeled zoledronic acid was added to the glass vial. After that the vial was vortexed for 1 min and incubated for 10 min at ambient temperature. Finally, the vial was centrifuged at 10,000 rpm for 5 min. The supernatant was separated and the radioactivity of the supernatant and precipitated hydroxyapatite particles was measured with a NaI well-type γ-counter and percentage binding was calculated.

Biodistribution studies

[^{99m}Tc]Tc-zoledronic acid (100 µL, 3.7 MBq) was injected intravenously to tail vein of rats. Groups of 3 rats each were sacrificed at different times (1, 2 and 4 h) post injection and the tissues and organs of interest were collected, immediately weighed and counted in a NaI well-type γ-counter. Subsequently, percentage uptake of radioactivity in each organ (bone, blood, lung, stomach, intestine, thyroid, liver, spleen, muscle and kidneys) was calculated as the percentage of the injected activity per gram tissue (%IA/g tissue). The total count injected per rat was determined by aliquot taken from the injected solution as a standard.

Scintigraphy

To evaluate all aspect of the whole body localization of [^{99m}Tc]Tc-zoledronic acid, planar scintigraphy was carried out using the single head gamma camera (small area mobile, Siemens, 140 keV high sensitivity parallel whole collimator and 10% window around 140 keV). Radio-complex (18.5 MBq, 100 µL) was administered to the rats and bone scanning was performed at different time point (1, 2 and 4 h) after injection. Before the imaging, rats were anesthetized with 0.05 ml ketamine 10% (3.3 mg) and 0.05 ml xylazine 2% (1.33 mg) intra-peritoneally.

RESULTS

Preparation and quality control

[^{99m}Tc]Tc-zoledronic acid (Figure 1) was prepared via the reduction of [^{99m}Tc]TcO4$^-$ by aqueous solution of stannous chloride and then its subsequent reaction with zoledronic acid. Amount of ligand was optimized in formulation and 100 µg (0.34 µmol) of zoledronic acid was the lowest amount, which reached a high labeling yield in preparation of radio-complex.

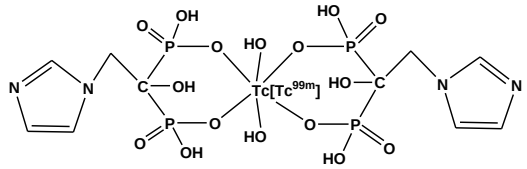

Fig 1. Proposed chemical structure of [^{99m}Tc]Tc-zoledronic acid

Stannous chloride was used to reduce [^{99m}Tc]Tc-pertechnetate to the lower oxidation state which enables [^{99m}Tc]Tc to react with the zoledronic acid as a ligand. A stable labeling with high radiochemical purity (>99%) was acquired with 100 µg (0.44 µmol) $SnCl_2$. Ascorbic acid was

used as an antioxidant and stabilizer of stannous ion. colloidal impurity in radio-complex was achieved in minimum amount along with [^{99m}Tc]Tc(IV)-ascorbate avoiding when 500 µg (2.8 µmol) ascorbic acid was used. The reaction pH evaluation showed that the best pH range for production of [^{99m}Tc]Tc-zoledronic acid with high labeling yield (>99%) was pH=1-2 and labeling yield decreased in higher pH range.

High labeling yield was achieved when optimized formulation (100 µg, 0.34 µmol zoledronic acid, 100 µg, 0.44 µmol $SnCl_2$, 500 µg, 2.8 µmol ascorbic acid and pH =1-2) was labeled with [^{99m}Tc] (555-2775 MBq) and was incubated at room temperature for 15 minutes. Although there is no idea what the structure exactly is, radiochemical purity of >99% was obtained while the maximal radioactivity of 2775 MBq was used for labeling (specific activity of 8050 MBq/µmol). In this way, a clear labeled product was obtained for further evaluation. Paper chromatography analysis of radio-complex showed that in the system of acetone as a solvent the Rf values were 0.9-1.0 for [^{99m}Tc]TcO4$^-$ and 0.0-0.1 for [^{99m}Tc]Tc-zoledronic acid and [^{99m}Tc]TcO2.χ H2O. In the system of phosphoric acid 15%, as a solvent [^{99m}Tc]Tc-zoledronic acid and [^{99m}Tc]TcO4$^-$ migrated with the solvent front with Rf values of 0.8-1.0 and [^{99m}Tc]TcO2.χ H2O remained at the origin (Rf = 0.0-0.1). The chromatography results showed unlabeled [^{99m}Tc]Tc ([^{99m}Tc]TcO4$^-$) and colloidal impurity ([^{99m}Tc]TcO2.χ H2O) were less than 1% and radiochemical purity for radio-complex was >99% (Figure 2).

The stability results which performed every 1 h showed that above complex prepared under optimal condition retaining a radiochemical purity >99% and >95% in saline and serum solutions for 6 h respectively. The radio-complex partition coefficient was calculated and found to be (log P) -2.68 which is a good indicator of its hydrophilicity. Hydroxyapatite binding Assay of [^{99m}Tc]Tc-zoledronic acid revealed a binding of 98% to hydroxyapatite particles. This shows that affinity of [^{99m}Tc]Tc-zoledronic acid toward hydroxyapatite remained in higher rate through formulation.

Biodistribution and imaging

Biological evaluation of [^{99m}Tc]Tc-zoledronic acid was performed in rat. As results in Table 1 show the kidneys uptake of radio-complex at 2 h post injection was 5.79±0.15 %IA/g which decreased to 3.69±0.08 %IA/g up to 4 h post injection. Liver uptake in all time point was low (0.34±0.06, 0.29±0.04 and 0.32±0.02 % IA/g at 1, 2 and 4 h post injection respectively). The blood uptake value was 0.94±0.15 % IA/g which decreased to 0.63±0.04 % IA/g in 4 h after injection. High level of activity up to 2.12±0.10 % IA/g at 1 h in bone was observed and this value
increased to a maximum of 4.53 ± 0.14 % IA/g at 2 h after injection. Bone to blood activity uptake ratio of radio-complex was 2.25 at 1 h and increased to 7.42 at 2 h. For over 4 h, bone uptake was 3.74±0.12 % IA/g while bone to blood uptake ratio was 5.93 with no significant concentration in any other organ and radioactivity was eliminated largely by the kidneys in urine.

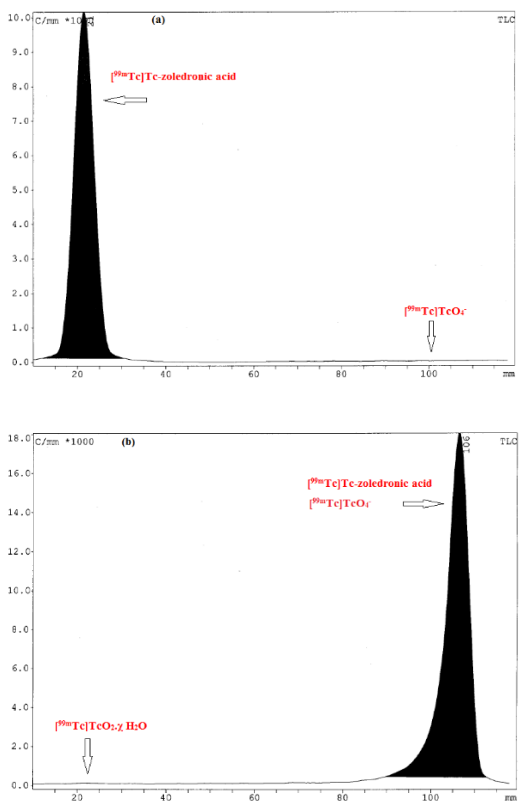

Fig 2. Whatman 1 paper radiochromatograms for [^{99m}Tc]Tc-zoledronic acid in acetone (a) and phosphoric acid 15% (b) as a mobile phases. Radio-complex showed radiochemical purity more than 99%. The impurities of [^{99m}Tc]TcO4$^-$ and [^{99m}Tc]TcO2.χ H2O were lower than 1%

The whole skeleton could be visualized through scintigraphy, which confirms the specific uptake of radioconjugate, by the bone. It was observed that the radioligand mainly was accumulated in the skeleton, kidney and urinary bladder. A clear image of the mice skeleton was obtained at 1, 2, 4 after injection of radio-complex (Figure 3).

Table 1. Biodistribution of [^{99m}Tc]Tc-zoledronic acid in rat at 1, 2 and 4 h after injection. Data are expressed as the percentage of injected activity per gram of tissue (% IA/g) and are presented as the mean ± SD (n = 4)

Organs	Post-injection time (h)		
	1	2	3
Blood	0.94 ± 0.15	0.61 ± 0.05	0.63 ± 0.04
Liver	0.34 ± 0.06	0.29 ± 0.04	0.32 ± 0.02
Stomach	0.17 ± 0.02	0.50 ± 0.01	0.34 ± 0.01
Spleen	0.23 ± 0.06	0.32 ± 0.02	0.25 ± 0.02
Intestine	0.29 ± 0.04	0.37 ± 0.02	0.41 ± 0.02
Kidney	3.17 ± 0.12	5.79 ± 0.15	3.69 ± 0.08
Bone (femur)	2.12 ± 0.10	4.53 ± 0.14	3.74 ± 0.12
Bone/Blood	2.25	7.42	5.93

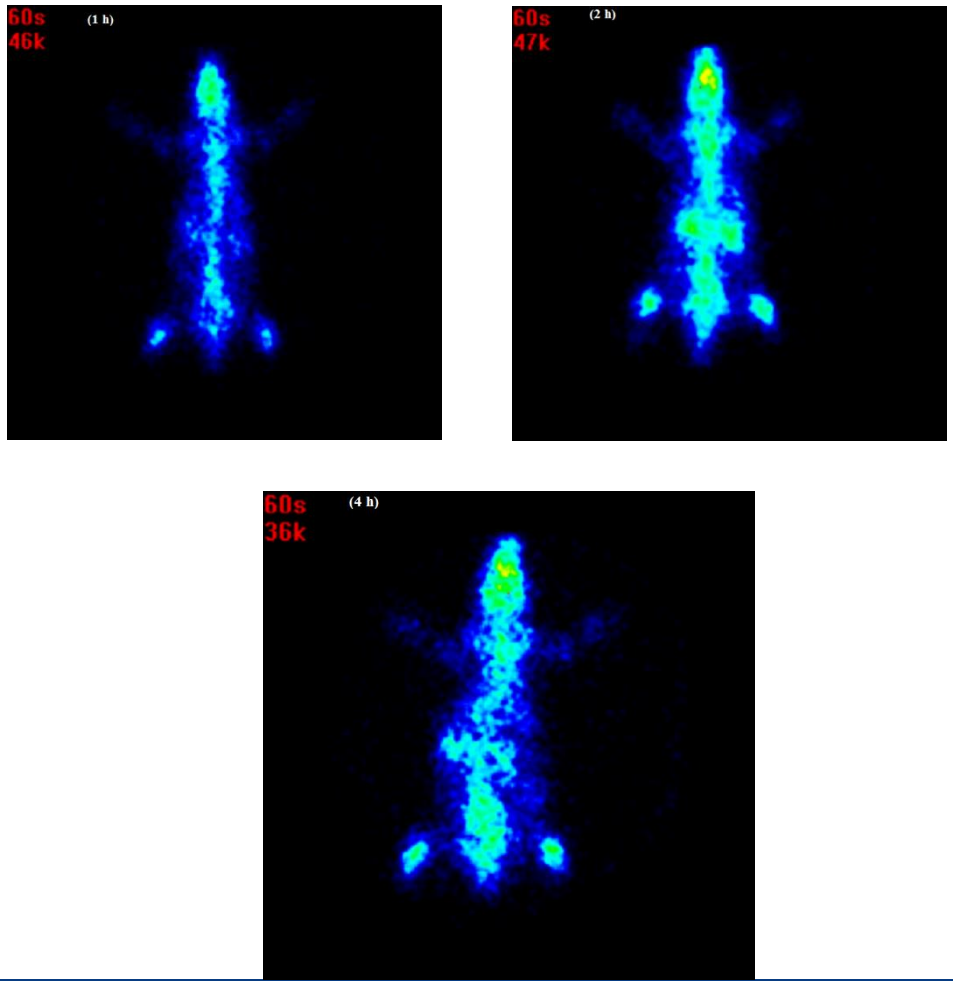

Fig 3. Whole body image in rat at different time point (1, 2 and 4 h) after injection of [^{99m}Tc]Tc-zoledronic acid. The total skeleton is clearly observable in different time point

DISCUSSION

In this study [^{99m}Tc]Tc-zoledronic acid was prepared with high specific activity and radiochemical purity. Adequate amount of ligand is necessary to achieve high radiochemical purity while high specific activity is maintained. Previously reported protocol in radiolabeling zoledronic acid involved 500µg ligand [11]. Due to the prolonged duration of action and its potentiality [17], we tried to reduce the ligand amount to 100 µg to prevent the probable pharmacological effects of the tracer as much as possible.

In [^{99m}Tc]Tc labeling chemistry, tin chloride plays an important role to reduce [^{99m}Tc]TcO4^{-} to lower oxidation state for reaction with ligand. However, as it could cause some hazardous effects including reproductive toxicity and the toxicity to some enzyme activities and oxidative damages [18] it is necessary to ensure an

optimum ratio of tin chloride. Compared to previously reported amount (400 µg) [11], in this study, $SnCl_2$ in the formulation was reduced to 100 µg while radio-complex was obtained in high purity impact. The addition of ascorbic acid is a safe and effective means of inhibiting the effect of oxygen and oxidants permitting the use of diagnostic kits containing low levels of $SnCl_2$ [19]. Therefore, the ascorbic acid used in our formula may also act as a protectant against the toxicity of $SnCl_2$. Intermediates like hydroxy and peroxy radicals are stabilized by the ascorbate through transferring H atom to the intermediate.

It is extremely important for radio-complex that isotope chelation remains stable as time passes. Our study showed that the radio-complex prepared through this formulation had high stability. Its stability could be contributed to optimization in amount of important components such as ligand and reducing agent. The labeling conditions (pH, reaction medium and incubation time) also played a decisive role in the achieved stability.

In comparison to [^{99m}Tc]Tc-MDP, the previously reported [^{99m}Tc]Tc labeled zoledronic acid administrated intravenously to the rabbits, there were no significant differences in the ratios of femur/Background [11]. The comparison of the bone uptake in the optimized formulation [^{99m}Tc]Tc-zoledronic acid and [^{99m}Tc]Tc-MDP, showed higher value for the former bone. The highest femur bone uptake value of [^{99m}Tc]Tc-zoledronic acid in rat was 4.53 %IA/g (2 h), and the minimum value was 2.12 %IA/g (1 h). The bone uptake of [^{99m}Tc]Tc-MDP in rat reached the maximum 1.77 %IA/g at 1 h. At 2 h post injection ^{99m}Tc-MDP exhibited lower level of accumulation in the rat femur bone than that of [^{99m}Tc]Tc-zoledronic acid (3.18 %IA/g versus 4.53 %IA/g). High bone uptake along with low liver uptake could help to acquire an image with more accuracy and lower background.

The generated images were well-defined using the optimized formulation for different components in preparing [^{99m}Tc]Tc-zoledronic acid. Based on the delayed images, with passing the time, better uptake of bone and increased target to background ratio was observed.

CONCLUSION

In this study, the formulation of a bone-imaging agent was optimized. [^{99m}Tc]Tc-zoledronic acid was prepared with high purity and stability under optimally adjusted conditions (0.34 µmol zoledronic acid, 0.44 µmol $SnCl_2$, 2.8 µmol ascorbic acid, 2775 MBq $^{99m}TcO4^-$ and pH =1-2). Significant bone uptakes along with fast elimination through kidneys were obtained for radio-complex. Considering these favorable properties, [^{99m}Tc]Tc-zoledronic acid can be introduced as a new bone imaging candidate.

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