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Synthesis, characterization, and in vivo skeletal localization of a new ^{99m}Tc-based multidentate phosphonate chelate: 5-Amino-1,3-bis(ethylamine-(*N*,*N* dimethyl diphosphonic acid) acetamido) benzene

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Abstract—The tetraphosphonate ligand, 5-amino-1,3-bis(ethylamine-(*N*,*N*-dimethyl diphosphonic acid) acetamido) benzene (IPTMP) used in the present study was prepared from 5-nitroisophthalate dimethylester to label with radionuclide for targeted diagnosis and therapy. The synthesized multidentate phosphonate ligand was characterized on the basis of spectroscopic techniques, which exhibited good metal ion control properties when complexed to ^{99m}Tc with high in vitro and in vivo stability. Excellent quality bone images of rabbit were imaged showing rapid clearance of background activity and visualization of skeleton at 1 h.

1. Introduction

Bone metastases occur frequently in patients with advanced breast, prostate, kidney, thyroid, and skin cancers. The challenge of treating bone metastasis is to deliver high doses of antitumor agents directly to tumor areas with minimal exposure to the rest of the body. This work is focused on development of the drug delivery to specifically target cancer within the skeleton where bone is being destroyed through the use of specific targeting molecules attached to the chelating agent that already has the affinity for bone. The utility of this work is that it could be used to target bone metastasis from any cancer. It is thus an object of the present work to provide a convenient synthetic route for the preparation of a new multidentate chelating agent to obtain more stable phosphonic acid based derivative for radiodiagnosis and radiotherapy.

Skeletal localization of phosphonate based bone imaging agents depends on several factors including bone–blood flow, enzyme activity (especially alkaline phosphatase), and active bone mineralization of hydroxyapatite crystals. In bone imaging we attempt to image the uncontrolled change of the mineralization process. Radiotracers have reported to be localized in these sites of increased mineralization activity in disease states. Amorphous calcium phosphate present in many bone lesions in high concentrations, which has a greater affinity for bone imaging agents, is also known to play a part in radionuclide accumulation.[1–4] The –OH on the phosphonate groups are suggestive of the bone matrix affinity.[5] There are other mechanisms of localization which involves calcium fixed to mitochondria rendering site for the deposition of the bone imaging agents are reported in the literature.[6–8] Phosphonic acid derivatives and more elaborate derivatives are widely used as complexing agents for calcium, zinc,[9] and/or a number of bivalent metals.[10] They are involved in a large number of biochemical processes and disease treatments[8] or diagnosis (scintigraphy in the case of ^{99m}Tc)[11–15] and therapy (^{90}Y, ^{153}Sm, and ^{188}Re).[18–20]

Thus, the binding of polyphosphates and bisphosphonates to calcified tissues is the basis for the use of these compounds as skeletal markers in nuclear medicine when linked to ^{99m}Tc. ^{99m}Tc-labeled compound used clinically in nuclear medicine are methylenediphosphonate (MDP), dicarboxypropanediphosphonate (DPD), hydroxyethylidine

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diphosphonate (HEDP), and ethylenediaminetetramethylene phosphonate (EDTMP).[14–17] Multidentate phosphonate derivatives containing more than two –C–PO_3–H_2 groups are effective chelating agents and are hydrophilic in nature. Greater number of phosphonate groups per molecule possess high chemical stability and produce 1:1 chelates than the conventional methylenediphosphonate. Due to the multiplicity of the ligands and the radionuclides now currently available, it is of interest to see the variation in bone uptake with the ^{99m}Tc complex of this new phosphonic acid derivative to develop suitable imaging agents to label with radionuclide having excellent radiophysical properties.

Phosphonates are synthesized from phosphorous acid by reaction with formaldehyde and either ammonia or amines with old methodology yielding only 20% of the required compound and resulted in the formation of bridge compound in higher yields (78%).[21] In this work new methodology is employed for producing phosphonate directly from carboxylate in one-step process making the route of synthesis much more convenient and producing high yield of phosphonate derivative.[22,23] Introduction of functionalized group to phosphonic acid derivative provides the reactive functionality for the conjugation of biological vector. The unique functionality of this phosphonic acid derivative departed considerably from the traditional acyclic phosphonate chelators used in nuclear medicine.

The multidentate agent was derived from *p*-NO_2-isophthalate ester and the NO_2 group was further reduced to reactive amine. The substitution of carboxylic acid pendant arms to phosphonic and the four nitrogens in the chelating system provides more strong coordination for metal–ion complexation. As is known, phosphonic acids are substantially more acidic than the corresponding carboxylic acids. As anticipated from the more polar nature of the phosphonic acid moiety, the phosphonic acids have appreciably lower log *P* values, indicating that they concentrate more favorably in the aqueous phase.[24]

Moreover, aminophosphonates show a propensity toward the strong chelation of metal ion.[10,25] In an effort to synthesize compounds that can be used in nuclear medicine applications, a new multidentate phosphonate derivative, 5-amino-1,3-bis(ethylamineacetamido)-benzene (IPTMP), was synthesized. The purpose was to prepare a synthetically useful phosphonic acid derivative with higher coordinating ability and a reactive moiety in the chelating system. Synthesis, radiolabeling, and biodistribution studies of a novel ^{99m}Tc-labeled multidentate phosphonate ligand IPTMP are described.

2. Results

2.1. Synthesis

The compound 5-amino-1,3-bis(ethylamine-(*N*,*N*-dimethyl diphosphonic acid) acetamido) benzene (IPTMP) was synthesized from 5-nitroisophthalate dimethyl ester and ethylenediamine (Scheme 1). The tetraacetic acid was formed by standard amine alkylation technique employing excess bromoacetic acid and aqueous 10 N NaOH by pH stat. 5-nitro-1,3-bis(ethylamine-(*N*,*N* dimethyl diphosphonic acid) acetamido) benzene (**3**) was prepared by the reaction of phosphorous acid in the presence of phosphotrichloride and characterized on the basis of spectral studies. The tetraphosphonate product was obtained in 89% yield and [$M+H^+$] was found to be 672.1 in positive mode. In ^{1}H NMR –CH_2–P chemical shift appeared at 3.59–2.88 ppm. Reduction of NO_2 group with Pd/C in basic media unmasked the amino group with a purity of above 97%.

2.2. Quality control

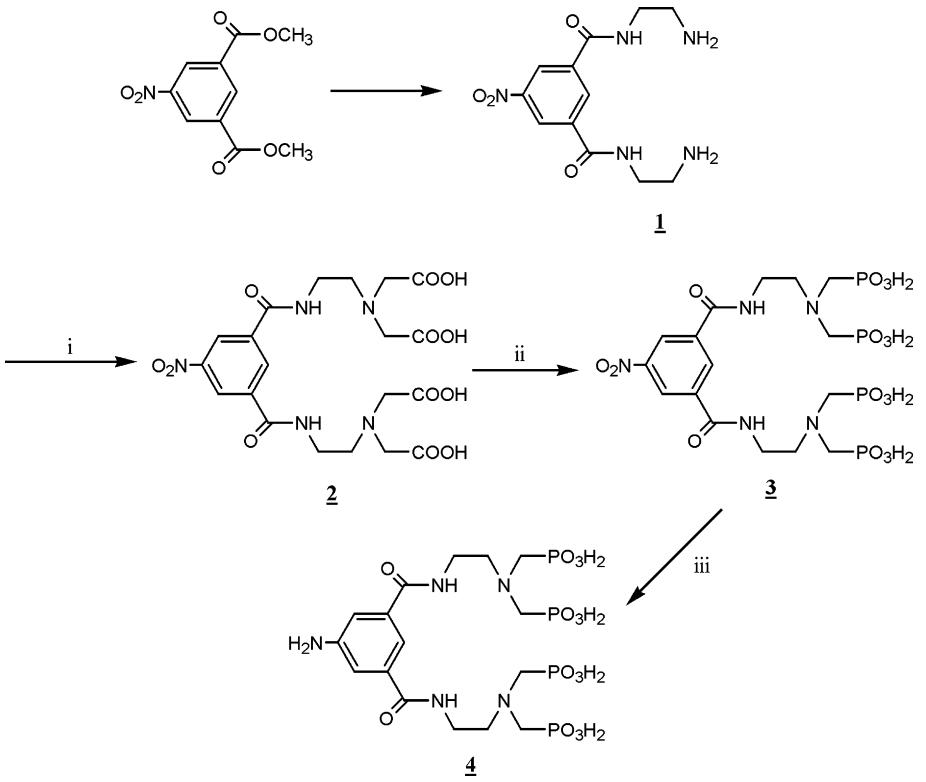
Radiochemical purity of ^{99m}Tc-IPTMP was estimated chromatographically using ITLC-SG (instant thin layer chromatography-silica gel) paper as the stationary phase and 100% acetone as the mobile phase. The complex remained at the point of spotting. Under identical condition $^{99m}TcO_4^-$ moved toward the solvent front. Thus the yield of free and complex ligand could be estimated. The labeling yield was found to be more than 97%. Metal-binding assay confirmed the radiochemical purity of IPTMP to be >97%. Phosphor imager showed only a single spot with $R_f = 0.3$ (10% ammonium acetate/methanol) which suggests the formation of only one species.

2.3. In vitro and in vivo stability studies

^{99m}Tc-IPTMP showed no transchelation toward serum proteins in vitro under physiological condition as only 2% detachment of the radiometal ion was observed at 24 h (Fig. 1). Radiolabeled MDP and IPTMP were challenged with DTPA (25–100 mM) (Fig. 2). Major transcomplexation of ^{99m}Tc to 25 mM DTPA was observed for ^{99m}Tc-MDP that was found to be 45%, whereas IPTMP showed only 6% transcomplexation of ^{99m}Tc to 25 mM DTPA. The blood activity curves in rabbit show that the blood clearance of ^{99m}Tc-IPTMP is comparable to that of ^{99m}Tc-MDP. The biological $t_{1/2}$ (*F*) was found to be 28 min and $t_{1/2}$ (*S*) was 8 h 25 min for ^{99m}Tc-IPTMP and $t_{1/2}$ (*F*) = 1 h 5 min; $t_{1/2}$ (*S*) = 8 h for ^{99m}Tc-MDP (Fig. 3). The initial clearance rate was comparable to that of MDP and also there was less activity present in the blood at 3 h post-injection. This accounts for high bone/blood and bone/muscles ratio exhibited by ^{99m}Tc-IPTMP.

2.4. Toxicity studies

The acute iv LD_{50} was determined to be 240 mg/kg. Doses <240 mg/kg were well tolerated and no adverse reaction was observed. Higher doses >240 g/kg were toxic and found lethal. At 108–180 mg/kg dose immediate adverse reaction was observed following tremors and increased heart rate, these reactions were observed to be gone completely after 2–3 min. No adverse signs were observed in these groups after 30 days. At 240 mg/kg dose 50% of the death was seen in 30 days. Two hundred and eighty milligrams per kilogram dose was found to be the lethal dose.



Scheme 1. Synthesis of 5-amino-1,3-bis(ethylamine-(*N*,*N* dimethyl diphosphonic acid) acetamido) benzene (**4**). Reagents: (i) $BrCH_2COOH$; (ii) H_3PO_3/PCl_3; (iii) 5% Pd–C/H_2.

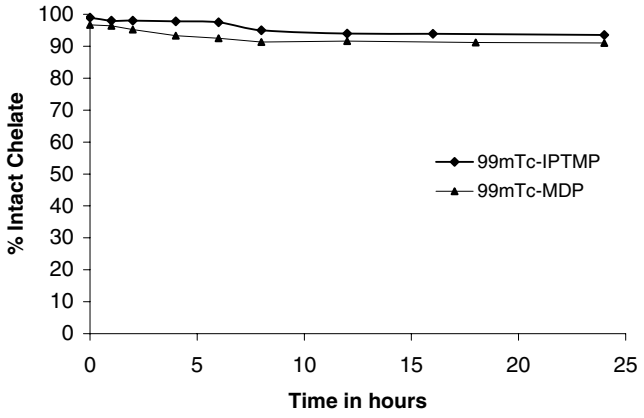


Figure 1. Human serum stability studies of ^{99m}Tc-IPTMP and ^{99m}Tc-MDP under physiological conditions.

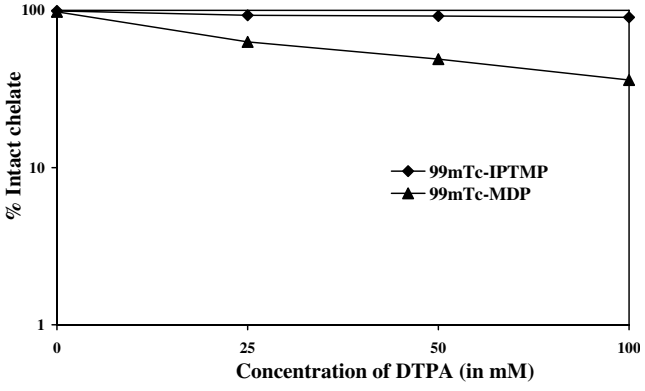


Figure 2. In vitro stability study of ^{99m}Tc-labeled MDP and multidentate chelate IPTMP challenged with DTPA (25–100 mM).

2.5. Scintigraphy

Scintigraphic images of ^{99m}Tc-labeled 5-amino-1,3-bis(ethylamine-(*N*,*N*-dimethyl diphosphonic acid) acetamido) benzene in rabbit in 3.3 kg rabbit showed rapid accumulation of radioactivity in bone. Imaging of animals was carried out at different time intervals after administering labeled compound intravenously. Early images were taken at 30 min and 1 h. Within 15 min labeled phosphonate ligand IPTMP accumulated in the shoulder region. After 1 h the ligand was localized in the whole skeleton of rabbit. A comparison study was conducted for the amino derivative (IPTMP) and MDP in normal rabbit of the same age and weight demonstrated comparable quality at 3 h with ^{99m}Tc-MDP and at 1 h with ^{99m}Tc-IPTMP post-injection. The scintigrams of ^{99m}Tc-IPTMP are of comparable quality to ^{99m}Tc-MDP scintigram in a 3.1 kg rabbit and the images also show that non-osseous tissue uptake of ^{99m}Tc-IPTMP is minimal and when assessed with its rapid blood clearance accounts for the low soft tissue activity (Fig. 4). In the early images of the ^{99m}Tc-IPTMP, major accumulation was seen in kidneys. This accounts for the aminophosphonate group in the compound, which has higher affinity for the kidney cells, but with the period of time it was cleared from the kidneys.

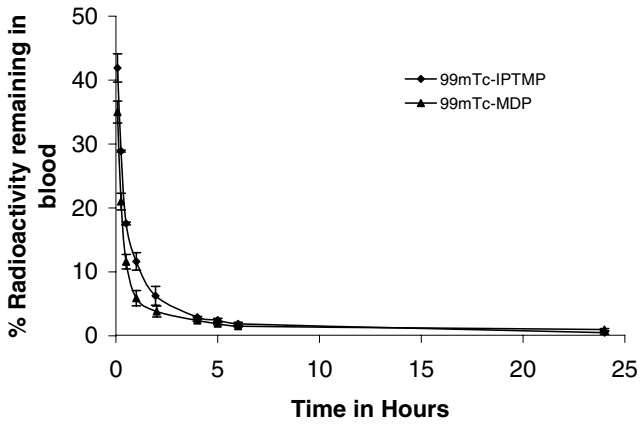


Figure 3. Blood clearance of ^{99m}Tc-labeled 5-amino-1,3-bis(ethylamine-(*N*,*N* dimethyl diphosphonic acid) acetamido) benzene in normal rabbit.

2.6. Biodistribution studies

In mice the skeletal uptake of ^{99m}Tc-IPTMP is higher at 1 h compared to ^{99m}Tc-MDP, which may be due to lesser structural forms and higher stability of complex. The bone uptake in mice from biodistribution was found to be 7.3 ± 0.69% ID/g at 4 h Table 1a. In biodistribution studies the tracer is noted to persist in the skeletal system even at 24 h and showed less accumulation in kidney. The excretion of the drug (IPTMP) is renal, therefore the accumulation in the kidneys showed high uptake (2.32 ± 0.01% ID/g) initially. But with the period of time it got cleared from the kidneys (0.142 ± 0.12% ID/g). This accounts for high bone/blood and bone/muscles ratio exhibited by ^{99m}Tc-IPTMP, which is comparable to ^{99m}Tc-MDP Table 1b.

3. Discussion

Entrapment of metal ion into a framework of multidentate poly (aminocarboxylates) such as bifunctional DTPA covalently attached to mAbs has been proven to provide thermodynamically stable compounds suitable for clinical trials.[26] Nevertheless, results obtained for human patients and for animal models indicate that the high level of radioactivity in blood, bone marrow, kidney, and liver limits the applicability of these compounds. This necessitates reduction of in vivo decomplexation of metal ions. The fast forming chelates like those of derivatives of EDTA undergo rapid transchelation to blood proteins.[26,27] Tetracarboxylate was prepared by the reaction of bromoacetic acid at high pH and converted to phosphonate by following reported

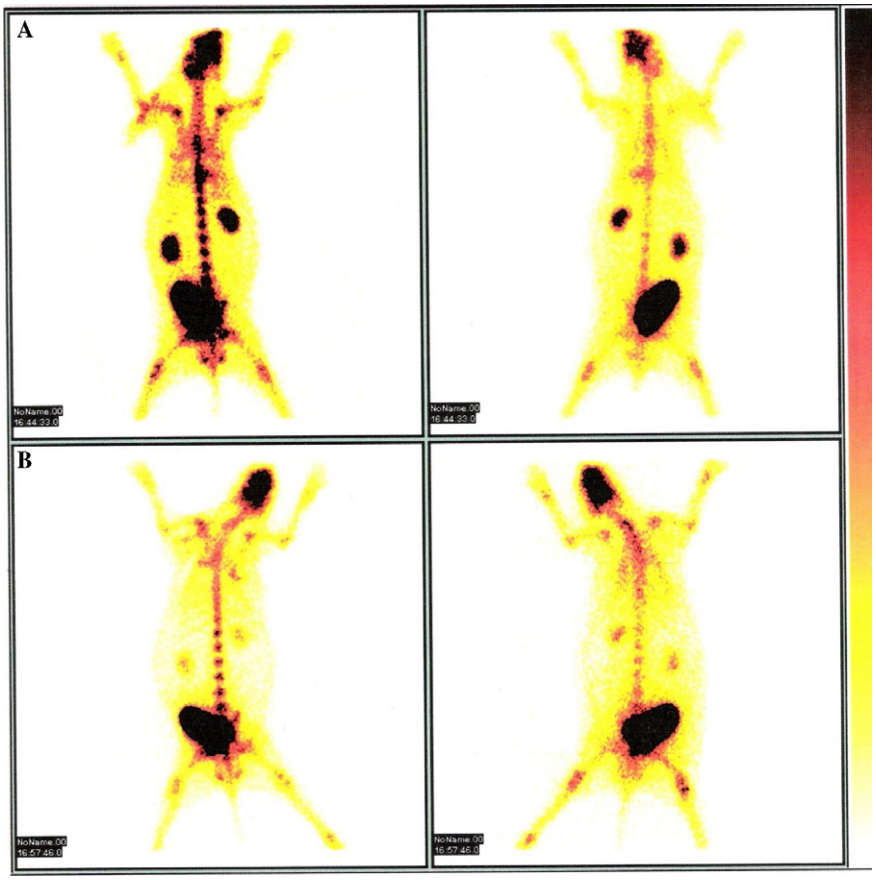


Figure 4. Whole body γ-Scintigraphic anterior and posterior images (A). ^{99m}Tc labeled 5-amino-1,3-bis(ethylamine-(*N*,*N* dimethyl diphosphonic acid) acetamido) benzene (1 h) (B). ^{99m}Tc-MDP in rabbit (3 h).

Table 1a. Biodistribution of ^{99m}Tc-labeled 5-amino-1,3-bis(ethylamine-(*N*,*N* dimethyl diphosphonic acid) acetamido) benzene in BALB/c mice

Organs	30 min % ID/g	1 h % ID/g	4 h % ID/g	24 h % ID/g
Blood	0.64 ± 0.01	0.71 ± 0.08	0.21 ± 0.06	0.037 ± 0.05
Heart	0.23 ± 0.01	0.21 ± 0.04	0.14 ± 0.09	0.087 ± 0.06
Liver	0.16 ± 0.01	0.14 ± 0.02	0.22 ± 0.08	0.038 ± 0.08
Lungs	0.16 ± 0.03	0.23 ± 0.06	0.21 ± 0.06	0.081 ± 0.03
Kidneys	2.32 ± 0.01	1.85 ± 0.12	2.47 ± 0.07	0.142 ± 0.12
Spleen	0.18 ± 0.08	0.16 ± 0.03	0.13 ± 0.02	0.128 ± 0.03
Stomach	0.12 ± 0.01	0.18 ± 0.06	0.26 ± 0.05	0.021 ± 0.02
Intestine	0.12 ± 0.05	0.13 ± 0.02	0.24 ± 0.01	0.06 ± 0.01
Bone	4.69 ± 0.92	6.6 ± 0.46	7.3 ± 0.69	2.96 ± 0.06
Muscles	0.22 ± 0.02	0.17 ± 0.09	0.13 ± 0.05	0.08 ± 0.01

Data from five animals expressed as % ID/g ± SD.

Table 1b. Comparative uptake (%ID/g) of ^{99m}Tc-multidentate chelates in mice

Organs	^{99m}Tc-MDP		^{99m}Tc-IPTMP	
	1 h	4 h	1 h	4 h
Blood	0.21 ± 0.01	0.19 ± 0.01	0.71 ± 0.08	0.21 ± 0.07
Kidneys	1.46 ± 0.20	0.98 ± 0.11	1.85 ± 0.12	2.47 ± 0.07
Bone	4.92 ± 0.15	5.92 ± 0.36	6.6 ± 0.46	7.3 ± 0.69

BALB/c mice, body weight = 22–28 g. Data from groups of five mice are expressed as mean % ID/g ± SD. Based on estimates assuming: blood volume = 7% of the body weight.

method of phosphonate synthesis. Phosphonate derivative produced in a Mannich type reaction by reacting ethylamines with phosphorous acid and formaldehyde leads to the formation of by-products which requires purification.[21] The new methodology describes one-step process for the synthesis of tetraphosphonate chelator using phosphorous acid and phosphorous trichloride; this eliminates the formation of unwanted intermediate product thereby obviating the need for its further purification.[22] The phosphonate derivative was synthesized using new methodology that gave product in high yield. Because of the four-methylene phosphonic acid groups/ligand molecule should form highly negatively charged chelates. The polydentate nature of the ligand having a reactive functionality for the conjugation of biological vector favors the formation of complex with only one ligand per metal atom as determined by metal binding assay which is required for in vivo stability and biological activity.[23] The multidentate agent developed included a *p*-NO_2 benzyl group for conjugation to biological targeting moieties, four nitrogens and four phosphonic groups for metal–ion chelation to wrap transition/lanthanide metal ions because most of these ions possess minimum six coordination number which cannot be fulfilled by MDP. To overcome the problem associated with MDP, this novel chelating system can be exploited as bone seeking agent for diagnosis and for therapeutic application since it has multidenticity to bind with β emitters.

Kunzmann et al. revealed a novel antitumor effect of aminobisphosphonates through their stimulation of γδT cells and the induction of an anti-multiple myeloma activity in patient samples. Therefore our compound being an aminophosphonate having several hydroxyl groups can afford high affinity toward bone matrix.[28] Different factors were considered before biodistribution study, particularly chelate stability in serum, which depends on various parameters such as medium pH, the metabolism of the metal, and the presence of binding proteins. The stability study for ^{99m}Tc-IPTMP showed no transchelation phenomena for the metal toward the physiological components of the blood because there was no release of radioactivity to complex in a more or less stable manner with albumin and transferrin and at blood pH form colloid substances with high affinity for carbonates, thus leading to sequestration in Reticulo-Endothelial tissue.[29,30] This study showed that ^{99m}Tc-IPTMP is highly stable in serum medium.

The blood activity curves in rabbits (Fig. 3) show that the blood clearance of ^{99m}Tc-IPTMP initially is faster than ^{99m}Tc-MDP due to which there was less activity present in the blood at 3 h post-injection. This accounts for high bone/blood and bone/muscles ratio exhibited by ^{99m}Tc-IPTMP, which is comparable to that of ^{99m}Tc-MDP. These findings in rabbits are an indication of the excellent in vivo stability of the ^{99m}Tc-IPTMP. The images also show that non-osseous tissue uptake of ^{99m}Tc-IPTMP is minimal and when assessed with its rapid blood clearance accounts for the low soft tissue activity. Rapid incorporation of phosphonates into calcified tissue leads to their short presence in the circulation.[1]

Biodistribution studies revealed, in mice the skeletal uptake of ^{99m}Tc-IPTMP is higher than ^{99m}Tc-MDP at 1 h, which may be due to lesser structural forms and higher stability of complex. 1:1 ligand ratio accounts for lesser structural forms and better-defined preparations of IPTMP, whereas MDP forms dimers, trimers, and oligomers with the metal ion. Four-phosphonate moiety in the same molecule as compared to MDP is responsible

for the formation of 1:1 complexation as shown by MBA. The relative uptake in rabbit is similar to the results obtained in mice from biodistribution.

Considering that the images and selective skeletal uptake of ^{99m}Tc-IPTMP is comparable to a commercial ^{99m}Tc-MDP agent (Fig. 4) suggests conjugation of IPTMP with targeting molecule and further optimization of the formulation (e.g., dose, quantity of compound, pH, Sn^{2+}, etc.) of ^{99m}Tc could produce even better results. For therapeutic application like octreotide, the kidneys can be blocked with non-specific agents to avoid any untoward reaction as it is being used in the case of petptide-based therapy.[31]

4. Conclusion

This investigation shows that tetramethylene phosphonate ligand obtained from *p*-nitroisophthalate is capable of forming stable ^{99m}Tc-chelate that exhibits high and selective in vivo bone uptake in both mice and rabbit. ^{99m}Tc-IPTMP has high bone uptake properties for skeletal imaging in terms of physiological and chemical stability. Its rate of blood clearance is rapid and its skeletal uptake is significantly higher at 1 h compared to that of ^{99m}Tc-MDP in mice and rabbits. These results certainly warrant further work with this ligand.

5. Experimental

5.1. Materials and methods

5.1.1. Chemicals. 5-Nitroisophthalate-1,3-dimethyl ester was purchased from Fluka, all the chemicals ethylenediamine, bromoacetic acid, phosphorous acid and phosphorous trichloride, stannous chloride dihydrate, 5% Pd/C, ethanol, and methanol (HPLC grade) were purchased from Sigma–Aldrich Co. ^{99m}Tc was procured from Regional Center for Radiopharmaceuticals (Northern Region), Board of Radiation and Isotope Technology (BRIT), Department of Atomic Energy, India. MDP kit is procured from CIS Bio International-Schering, France.

5.1.2. Instrumentation. The ^{1}H NMR spectra were recorded by Bruker 300 MHz system. Mass spectrum was obtained inhouse from ion trap SL 1100 system (Agilent, Waldbronn, Germany) using ESI positive and negative mode.

5.1.3. Animal models. Animal protocols have been approved by Institutional Animal ethics Committee. New Zealand Rabbits 2–3.5 kg and BALB/c mice 22–28 g were used for blood clearance, imaging, and biodistribution studies.

5.2. Synthesis

5.2.1. Synthesis of 5-nitro-1,3-bis(ethylenediamine) (1). Dimethyl-5-nitroisophthalate (1 g; 4.18 mmol) was dissolved in neat ethylenediamine at 0 °C under nitrogen atmosphere and the reaction mixture was stirred for 24 h. EDA was removed under reduced pressure. The obtained oily product was dissolved in chloroform and allowed to evaporate slowly at room temperature.

Yield: 96.8%; IR (KBr) ν cm^{-1}: 3367, 3086, 1654, 1534, 1487, 1348. ^{1}H NMR (300 MHz): δ 8.76 (2H, Ar, s); 8.62 (1H, Ar, s); 3.42 (4H, t); 2.81 (4H, t). Mass: MS$^+$ *m*/*e* Calcd 295.2, found [M+H$^+$] 296.26. Elemental Anal. Calcd for $C_{12}H_{17}N_5O_4$: C, 48.81; H,5.80; N,23.72; O, 21.67. Found: C, 48.80; H, 5.81; N, 23.71; O, 21.68.

5.2.2. Synthesis of 5-amino-1,3-bis(ethylamine (*N*,*N*-diacetic acid) acetamido) benzene (2). Bromoacetic acid (2.12 g; 15.25 mmol) was added to a stirring solution of **1** (1 g; 3.37 mmol) in 15 mL of distilled water at room temperature, pH 5. After complete addition, the temperature was raised to 70 °C and pH was maintained at 10 by the solution of 0.1 M NaOH.

Yield: 89% IR (KBr) ν cm^{-1} 3435, 3013,2850, 1634, 1533, 1451, 1355, 1199, 1145, 722. ^{1}H NMR (300 MHz) δ 8.74 (2H, Ar, s); 8.51 (1H, Ar, s); 3.32 (12 H, m); 2.66 (4H, t). Mass: MS$^+$ *m*/*e* Calcd 528.15, found [M+H$^+$] 528.12, (M+2Na−H) 570. Elemental Anal. Calcd for $C_{20}H_{25}N_5O_{12}$: C, 45.54; H, 4.78; N, 13.28; O, 36.40. Found: C, 45.53; H, 4.78; N, 13.27; O, 36.42.

5.2.3. Synthesis of 5-nitro-1,3-bis(ethylamine-(*N*,*N* dimethyl diphosphonic acid) acetamido) benzene (3). To a solution of **2** (0.5 g, 0.948 mmol) dissolved in 10 mL of toluene added phosphorous acid (0.38 g, 4.77 mmol) under nitrogen atmosphere. The reaction mixture was refluxed while phosphorous trichloride in a volume (0.65 g, 4.74 mmol) was added dropwise to the refluxing mixture. At the end of 3 h toluene was removed following addition of water. The reaction mixture was filtered and concentrated under vacuum. The concentrated product was precipitated on addition of ethanol.

Yield: 82% IR (KBr) ν cm^{-1} 3382, 2999, 2445, 1644, 1542, 1464, 1180, 1014, 939, 530. Mass: MS$^+$ *m*/*e* Calcd 671.2, found [M+H$^+$] 672.1, (M+Na−H) 694.1. ^{1}H NMR (300 MHz) δ 8.78 (2H, Ar, s); 8.56 (1H, Ar, s); 3.34 (4H, t); 2.62 (4H, t), 2.4 (8H, m) Elemental Anal. Calcd for $C_{16}H_{29}N_5O_{16}P_4$: C, 28.63; H, 4.35; N, 10.43; O, 38.13; P, 18.46. Found: C, 28.62; H, 4.36; N, 10.43; O, 38.11; P, 18.48.

5.2.4. Synthesis of 5-amino-1,3-bis(ethylamine-(*N*,*N* dimethyl diphosphonic acid) acetamido) benzene (4). To a solution of (**3**) (100 mg) in water flushed with nitrogen was taken into schlenk flask under vacuum. Molar equivalent of 5% Pd/charcoal in nitrogen flushed, water was added and fitted onto an atmospheric hydrogenator. The apparatus was flushed with H_2 (g) twice to fully saturate the catalyst. The hydrogenation was allowed to proceed until the uptake of H_2 (g) had reached saturation. The reaction mixture was filtered through a bed of Celite. The filtrate was reduced to dryness by rotary evaporation.

Yield: 86% IR (KBr) ν cm^{-1} 3540, 3350, 3180, 1658, 1551, 1430, 1182, 1010, 928, 510. ^1H NMR (300 MHz) δ 8.2 (2H, Ar, s); 7.4 (1H, Ar, s); 3.30 (4H, t). 2.4 (8H, m) ESI-MS: Calcd *m/e* 641.3 for $C_{16}H_{31}N_5O_{14}P_4$; found $[M+H^+]$ 642.1, Elemental Anal. Calcd for $C_{16}H_{31}N_5O_{14}P_4$: C, 29.96; H, 4.87; N, 10.92; O, 34.93; P, 19.32. Found: C, 29.95; H, 4.88; N, 10.93; O, 34.92; P, 19.31.

5.3. Radiochemical synthesis of ^{99m}Tc-IPTMP

Phosphonic Acid derivative (10.0 μmol) was taken and stannous chloride 1.0 μmol was added. The pH of the reaction mixture was adjusted to 6.0–6.5 with 0.5 M Na_2CO_3. The mixture was passed through a 0.22 μm Millipore filter into a sterile vial. ^{99m}Tc Eluate containing 400–800 MBq activity was added and the complex was incubated for 30 min at room temperature to get optimum labeling yield. The labeling efficiency was estimated chromatographically using ITLC-SG (instant thin-layer chromatography-silica gel) paper as the stationary phase and 100% acetone as the mobile phase. Percentage of colloids was determined using pyridine/acetic acid/water (3:5:1.5) as the mobile phase and ITLC-SG strip as stationary phase. In vitro stability of the complex was determined chromatographically by estimating labeling efficiency at different time intervals up to 24 h.

The complexation was determined by titration of the chelating groups with ^{111}In. A stock solution of carrier-free $^{111}InCl_3$ (20 μL, in sodium acetate buffer, pH 5.5, adjusted with 1 M sodium acetate) was added to a stock solution (20 μL of 50 mM, 0.1 μmol). Then 16 μL of 0.1 M sodium acetate buffer, pH 7, was added. The pH was checked to be 7. After 3 h, the mixture was analyzed by using a TLC system (1:1 MeOH/10% NH_4OAc).

5.3.1. DTPA challenge. ^{99m}Tc-MDP and ^{99m}Tc-IPTMP (300 MBq) were incubated at different concentration 25, 50, and 100 mM DTPA, and maintained at 37 °C upto 24 h. Periodically, samples were removed, spotted on a 10-cm ITLC-SG strip, and developed in 0.9% NaCl. Once the solvent front had reached the end of the strip, it was removed from the solvent and cut at an R_f of 0.5. The two portions were assayed for radioactivity, and the amount of intact chelate was determined.

5.4. Animal studies

5.4.1. Toxicity studies. Toxicity study was carried out in groups of 5 male BALB/c rats. In the experiment, a single dose of IPTMP was administered iv into groups of five male BALB/c mice of 22–28 g. The dosage levels were 30–280 mg/kg body weight. The animals are weighed weekly and observed for clinical signs of toxicity and number of deaths that occurred in 4 weeks was recorded after treatment.

5.4.2. Blood clearance. In normal rabbit weighing about 2–2.5 kg, 10 MBq of the ^{99m}Tc-IPTMP was administered intravenously through the dorsal ear vein of the animal. At different time interval starting from 5 min to 24 h persistence of activity in terms of percentage-administered dose in samples at different time intervals was calculated using gamma counter.

5.4.3. Bone scintigraphy. Imaging of rabbit was done at different time intervals after administering 10 MBq of the ^{99m}Tc-IPTMP intravenously in normal rabbit. A comparison study was carried out by administerting ^{99m}Tc-MDP in rabbits and scintigrams of both the radiotracers were compared at different time points.

5.4.4. Biodistribution studies. This parameter was studied in male BALB/c mice (22–28 g). An aliquot of 3.7 MBq in each mouse was injected intravenously through the tail vein. The animals were sacrificed at 1, 4, and 24 h post-injection. The animals were dissected and the various organs were removed, made free from adhering tissue, weighed, and radioactivity was measured in each organ. The data were expressed as percent administered dose per gram of the organ.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2006.10.005

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