



Radiolabeling, Quality Control, and Cell Binding Studies of New ^{99m}Tc-Labeled Bisphosphonates: ^{99m}Tc-Ibandronate Sodium

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ABSTRACT

Objectives: Early detection of bone cancer is critical for treating symptoms, minimizing pain, and increasing overall quality of life. It is critical to develop novel radiopharmaceuticals with high labeling efficiency and stability for the diagnosis of bone cancer. This research aims to design a novel radiopharmaceutical that may be used to diagnose bone cancer.

Materials and Methods: In this study, ibandronate sodium (IBD), a bisphosphonate analog, was radiolabeled with technetium-99m [^{99m}Tc] and quality control tests on the newly developed radiopharmaceutical ([^{99m}Tc]Tc-IBD) were performed using radioactive thin layer chromatography. After that, the incorporation of [^{99m}Tc]Tc-IBD into hydroxyapatite (HA) crystals and a human bone osteosarcoma cell line (U2OS) was tested.

Results: According to the results obtained, optimal radiolabeling procedure was obtained for [^{99m}Tc]Tc-IBD with 200 µg.mL⁻¹ IBD, 20 µg stannous chloride, and ^{99m}Tc with 37 MBq radioactivity. The reaction mixture was adjusted to pH 5.5 and incubated at room temperature for 15 min. The radiochemical purity of [^{99m}Tc]Tc-IBD was found to be greater than 95% at room temperature for up to 6 h. Additionally, chromatography analysis showed >95% [^{99m}Tc]Tc-IBD complex formation with promising stability for up to 24 h in saline and up to 2 h in cell medium. The percentage binding of IBD to HA was 83.70 ± 3.67 and the logP of [^{99m}Tc]Tc-IBD was -1.1014. The radiolabeled complex exhibited a higher rate of cell incorporation to U2OS cells compared to Reduced/Hydrolyzed ^{99m}TcO₄⁻.

Conclusion: The newly produced radiopharmaceutical is very promising according to the results of *in vitro* cell culture, HA binding, and quality studies, and will be a step forward for further studies in nuclear medicine for bone cancer diagnostics.

Key words: Ibandronate sodium, technetium-99m, radiopharmaceuticals, radiolabeling, bone cancer cells

INTRODUCTION

Bisphosphonates (BPs) are a group of medications used to help prevent and treat bone loss.¹ They are structural counterparts of pyrophosphate molecules that occur, when the oxygen atom in the phosphorus-oxygen-phosphorus (P-O-P) bond in pyrophosphates is replaced with a carbon atom, resulting in the formation of a phosphorus-carbon-phosphorus (P-C-P) bond.² Since BPs bind to hydroxyapatite crystals (HA), they have a high affinity for bone minerals, like their natural analog pyrophosphates. Non-nitrogen-containing BPs (non-N-BPs) and nitrogen-containing BPs (non-N-BPs) are the two types of BPs (N-BPs). Ibandronate (IBD) is a very powerful oral N-BP that comprises a core P-C-P structure that is required for binding

to the mineral surface of the bone and prevents osteoclast-mediated bone resorption.^{2,3} IBD's main pharmacological impact on bone tissue is due to its affinity for HA, which is a component of bone's mineral matrix.⁴ The bone affinity of IBD is significantly high because of phosphonate groups and the chemical structure of IBD is ((1-hydroxy-3-[methyl(pentyl) amino] propane-1,1-diyl)bis (phosphonic acid)).⁵ The possible schematic design of the direct radiolabeling method for [^{99m}Tc] Tc-IBD is shown in Figure 1.

Early and accurate identification of bone cancer, followed by proper therapy, is crucial for managing symptoms and extending life expectancy. Because radiolabeled BPs are used as bone-seeking radiopharmaceuticals, bone scintigraphy has

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become one of the most extensively used diagnostic nuclear medicine procedures, providing earlier diagnosis or detecting more lesions than conventional radiographic approaches.⁶⁻⁹

In the past decades, several BPs compounds such as alendronate (ALD), risedronate, and zoledronate were radiolabeled with technetium-99m [^{99m}Tc] for use as a bone imaging agent to diagnose various clinical diseases that typically result in widespread skeletal metastases.¹⁰⁻¹⁴

In radiopharmaceuticals, there are radioactive and pharmaceutical parts together. A suitable radionuclide for radiolabeling experiments can be determined by considering aspects including radiation dose, cost, and availability. ^{99m}Tc has recently become the most widely used radionuclide for labeling research.¹⁵ By many procedural advantages related to the physical properties of this isotope, the use of ^{99m}Tc may enhance the quality of images and radiation protection for patients and staff.¹⁶ ^{99m}Tc is a radionuclide with monoenergetic gamma rays of 140 KeV, with a half-life of 6 h, and diverse chemistry for forming complexes.¹⁷

Although animal experimental studies are important for cancer detection, *in vitro* cell culture research was used to assess various cancer binding affinities. Many researchers prefer to do binding studies using cell lines before performing animal experiments, when developing a new radiopharmaceutical for imaging.¹⁸⁻²⁴ In this procedure, radiolabeled pharmaceuticals or formulations are treated to various types of cells, and then, the radioactivity in the cells and cell medium is measured at various time intervals.

This study aimed to develop a novel radiopharmaceutical for use in the detection of bone cancer. IBD was radiolabeled with ^{99m}Tc for this purpose. Labeling efficiency and stability of the compound were studied. Cell incorporation tests were used to assess the newly produced radiopharmaceutical's binding affinity to cancer cells. In human bone osteosarcoma cell line, incorporation of [^{99m}Tc]Tc-IBD to cancer line was examined as part of cell culture research (U2OS).

MATERIALS AND METHODS

Materials

Roche (Germany) provided IBD, while Merck (Germany) provided stannous chloride and ascorbic acid as well as all the solvents. Molybdenum-99 (^{99}Mo)/ ^{99m}Tc generator produced $^{99m}\text{TcO}_4^-$ (Ege University, Türkiye). Pall Life Science provided 0.22 μm pore size syringe filter. Gibco Invitrogen (Grand Island, NY) provided cell culture products and supplies. American Type Culture Collection (ATCC) provided the U2OS cell line. All other solvents were of analytical quality and were not purified further. A dose calibrator (AtomLab 100), a TLC scanner (BioScan AR 2000), and a gamma counter (Sesa Uniscaller) were used to count radioactive samples.

Radiolabeling studies

Varying amounts of reducing agent were used to test the radiolabeling of [^{99m}Tc]Tc-IBD. With ascending radioactive thin layer chromatography (RTLC) studies, radiochemical purity (RP) was assessed. To obtain the best outcome, the role of

antioxidant agent amount, incubation time, radiation dose, pH, and filtration parameters were examined.

Effect of reducing agent amount on labeling

$^{99m}\text{TcO}_4^-$ was eluted from $^{99}\text{Mo}/^{99m}\text{Tc}$ generator in +7 oxidation level. For ^{99m}Tc , this level is unable to label with any component or formulation when added directly. Before the radiolabeling, ^{99m}Tc must be reduced to lower oxidation levels to form complexes with the ligand and synthesize the radiopharmaceutical. For this purpose, various reduction agents, such as stannous chloride, stannous tartrate, sodium borohydride, sodium dithionite, hydrohalic acids, formamidine, sulfonic acid, and others, have been employed by the researchers. Because of its non-toxic and stable properties, stannous chloride is widely used as a reductant within them.²⁵

The role of reducing agent quantity on labeling was tested using stannous chloride in this study. IBD (100 μg) was dissolved in 0.5 mL of 0.9% sodium chloride solution (SF). Under the influence of a bubbling nitrogen environment, stannous chloride was added to the stock solution. $^{99m}\text{TcO}_4^-$ was reduced using different amounts of stannous chloride (5, 10, 20, 50, and 100 μg) at neutral pH (pH: 7.0) (1 mg reducing agent diluted in 1 mL pure water). In 0.1 mL SF, 37 MBq $^{99m}\text{TcO}_4^-$ was used for radiolabeling and before the radiochemical testing, the [^{99m}Tc]Tc-IBD was left at room temperature for 15 min.

Effect of antioxidant agent amount on labeling

Auto radiolysis occurs in ^{99m}Tc radiopharmaceuticals at all stages of production, including preparation, release, and storage. As a result, it is critical to use a stabilizer to prevent radiopharmaceuticals' auto radiolysis. Antioxidant agents like ascorbic acid, gentisic acid, and *p*-aminobenzoic acid, which quickly react with hydroxyl and superoxide radicals, have been used as stabilizers to reduce ^{99m}Tc radiopharmaceutical radiolytic disintegration.²⁶

Ascorbic acid as an antioxidant agent was used to investigate the effect of the quantity of antioxidant agent on labeling in this study. Radiolabeling experiments were carried out in the presence of ascorbic acid and in the absence of ascorbic acid. In 0.5 mL SF, IBD (100 μg) was dissolved. Each of the three vials was added 20 μg of stannous chloride. Then, before labeling, 1 and 2 μg of ascorbic acid were added to two vials, respectively, while no ascorbic acid is added to the 3rd vial. 37 MBq/0.1 mL of $^{99m}\text{TcO}_4^-$ was used for radiolabeling. The labeling efficiency was determined using RTLC after a 15 min incubation period.

Effect of incubation time on labeling

To generate a complex with high labeling efficiency and stability, radiopharmaceuticals should be left to stand for a while after labeling. RP of the [^{99m}Tc]Tc-IBD was studied by RTLC tests at 5, 15, 30, 45, and 60 min post-labeling to examine the incubation time.

Effect of radioactivity doses on labeling

Because of the personnel's and the area's radiation safety, *in vitro* radiolabeling investigations were performed with 37 MBq of $^{99m}\text{TcO}_4^-$. Also, the RP of [^{99m}Tc]Tc-IBD was investigated with 185 and 370 MBq radiation doses in addition to the 37 MBq radiation

dose because human research including radiopharmaceuticals require higher radiation doses.

Effect of pH on labeling

For stability and integrity, all radiopharmaceuticals should have a suitable pH value. The ideal pH of a radiopharmaceutical is 7.4 (pH of the blood), yet, it can vary between 2.0 and 9.0 due to the blood's high buffering capacity.²⁰ Although [^{99m}Tc]Tc-IBD should be compatible with body pH, small-volume preparations are adjusted to pH stable.

In this work, using 0.1 N NaOH and HCl, the role of pH on RP of [^{99m}Tc]Tc-IBD was investigated for pH 4.0-7.4.

Effect of filtration on labeling

Because radiopharmaceuticals are administered *via* the parenteral route, they must be sterilized using the appropriate procedure. Filtering with a membrane filter with a particle size of 0.22 μm enables sterile filtration for radiopharmaceuticals, while also removing reduced/hydrolyzed (R/H) $^{99m}\text{TcO}_4^-$ from the medium to improve image quality.

Two batches of radiopharmaceuticals were prepared with only 20 μg of stannous chloride to see whether filtration had any effect on labeling. While one group was filtered after a 15 min incubation period, the other was not. A RTLC scanner was used to assess the labeling stability of the two groups.

In vitro stability

After ^{99m}Tc labeling, IBD was left in SF for 24 h at 25°C and in cell medium for 2 h at 37°C. RTLC experiments at predetermined time intervals were used to evaluate the complex's labeling stability.

Stability of [^{99m}Tc]Tc-IBD in SF

To 0.9 mL SF, 0.1 mL of [^{99m}Tc]Tc-IBD reaction media was added. For 24 h, the mixture was incubated at 25°C. To test the stability of [^{99m}Tc]Tc-IBD in SF, one drop of radiolabeled solution was spotted on chromatography papers at various times (0, 1, 2, 3, 4, 5, 6, and 24 h) and RTLC experiments were carried.

Stability of [^{99m}Tc]Tc-IBD in cell medium

[^{99m}Tc]Tc-IBD reaction medium (0.1 mL) was mixed with 0.9 mL Dulbecco's modified essential medium (DMEM) containing 10% fetal bovine serum (FBS). The radiolabeled solution was incubated for 2 h at 37°C. To determine the stability of [^{99m}Tc]Tc-IBD in cell medium, 2-3 μL of sample was spotted on chromatographic paper at pre-determined times (0, 30, 60, and 120 min) and RTLC studies were performed.

RTLC procedure

In RTLC studies, ITLC-SG chromatographic sheets were used as stationary phase. In [^{99m}Tc]Tc-IBD solution, free $^{99m}\text{TcO}_4^-$ and R/H $^{99m}\text{TcO}_4^-$ were determined by 100% acetone and acetonitrile/water/trifluoroacetic acid (ACN/W/TFA; 50/25/1.5) solvent system as the mobile phase. The radioactivity of the sheets was assessed *via* a TLC scanner and the RP (%) of [^{99m}Tc]Tc-IBD was determined using equation (1) by subtracting the sum of the detected impurity percentages from 100:

$$\text{RP (\%)} = 100 - [\text{free } ^{99m}\text{TcO}_4^- (\%) + \text{R/H } ^{99m}\text{TcO}_4^- (\%)] \quad (1)$$

Cell culture studies

U2OS cell line was grown in DMEM solution supplemented 10% FBS. 3×10^5 cells were seeded in six wells with a transwell insert filter to form cell monolayers. The cells were kept at 37°C, 90% humidity, and 5% CO_2 . The adherent cells were split daily with trypsin/ethylene diamine tetraacetic acid in a 1:20 ratio.

In vitro incorporation studies

In vitro incorporation tests with the U2OS cells were used to compare the incorporation of [^{99m}Tc]Tc-IBD and R/H $^{99m}\text{TcO}_4^-$ to human bone osteosarcoma cells. For this experiment, cells were incubated with 3.7 MBq [^{99m}Tc]Tc-IBD for 30, 60, and 120 min at 37°C. The samples were collected from the cell medium and placed in tubes after the incubation time. Adherent cells were also trypsinized, washed in phosphate buffered saline (PBS) once, and transferred to tubes. A gamma counter was used to count the amount of radioactivity in the cell media and U2OS cells. The cellular uptake was calculated by dividing the total activity counted by the proportion of activity counted in the cells. The following equation, (equation 2), was used to determine the radioactivity (%) of cells.

$$\text{Radioactivity of cells (\%)} = (\text{radioactivity of cells/total radioactivity}) \times 100 \quad (2)$$

Hydroxyapatite-binding studies

HA binding test was performed with only minor changes to the procedure previously described.⁹ In a short, HA (5 mg, synthetic, powder) was added to PBS (1 mL) at the pH of 7.4. After that, 10 μL (0.5 MBq) of [^{99m}Tc]Tc-IBD were added to each HA solution, and the suspensions were shaken for 1 h at 37°C. A 50 μL sample of each sample's supernatant was tested for radioactivity in a gamma counter after 10 min of 5000 rpm centrifugation. The same process was used to obtain and count a control sample that did not contain HA. The following equation 3 was used to calculate the percentage of HA binding:

$$\% \text{ HA binding} = [1 - (\text{radioactivity of sample/radioactivity of control})] \times 100 \quad (3)$$

Biological tests of [^{99m}Tc]Tc-IBD

Because radiopharmaceuticals are applied to people, they must be sterile, isotonic, and free of pyrogens. The sterility, isotonicity, and pyrogenicity of [^{99m}Tc]Tc-IBD were tested for this purpose.

Sterility test

Sterility of the IBD solution was determined using the British Pharmacopoeia's direct inoculation method.²⁷ The IBD solution was inoculated aseptically into the sterilized terrific broth medium and tryptic soy broth tubes and incubated for 7 days at $35 \pm 2^\circ\text{C}$. The growth of microorganisms in the tubes was assessed at the end of the incubation period.

Isotonicity test

The IBD solution's isotonicity was determined using an osmometer. The calibrated instrument was used to analyze the samples in eppendorf tubes.

Pyrogenicity test

In a bacterial endotoxins test, pyrogenicity of the IBD solution was determined using the gel-clot technique. In terms of pyrogenicity, pyrogenicity of the prepared IBD solution and the standard endotoxin solution was compared. According to the European protocol, Limulus amoebocyte lysate test was performed to validate radiopharmaceutical preparation.^{27,28}

Lipophilicity studies

For lipophilicity tests of ^{99m}Tc -IBD, *n*-octanol and PBS (pH: 7.4) were used. In a centrifuge tube, 500 μL *n*-octanol, 500 μL PBS (pH: 7) and 150 μL ^{99m}Tc -IBD were mixed and centrifuged at 2500 rpm for 30 min. The mixture was separated into two phases after centrifugation. A gamma counter was used to count 100 μL of each phase activities. This process was carried out for four times.²⁰

Statistical analysis

Microsoft Excel was used to calculate the means and standard deviations (SD). The *t*-test was used to evaluate statistical significance. Differences were considered significant at 95 percent confidence level ($p > 0.05$). Unless otherwise noted, all experiments were carried out in triplicate. The results are presented as a mean with SD.

RESULTS

Radiolabeling studies

Our research group developed the radiolabeling of ^{99m}Tc -IBD. The best results were obtained by experimenting with radiolabeling parameters.

Effect of reducing agent amount on labeling

Labeling tests were carried out to determine how the amount of reducing agent affected the labeling yield. The labeling yield was low, and the chromatograms were irregular at lower

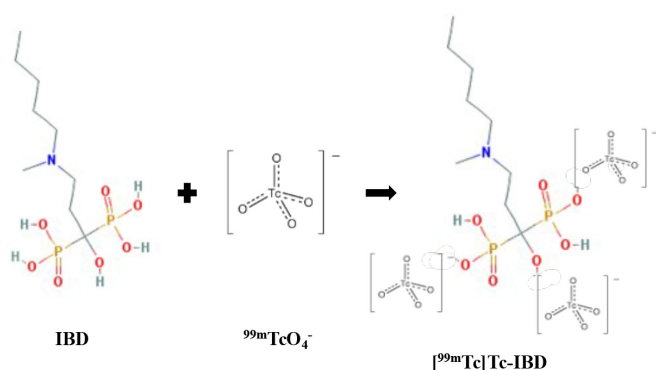


Figure 1. The possible direct radiolabeling approach for ^{99m}Tc -IBD

^{99m}Tc : Technetium-99m, IBD: Ibandronate sodium

concentrations of stannous chloride than the optimal value. The labeling yield was significantly decreased by increasing the reducing agent concentration above the optimal limits. In this study, RP (%) of formulations containing various concentrations of stannous chlorides is shown in Figure 2.

The amount of reducing agent that was shown to be the most effective was 20 μg , according to the findings. Labeling efficiency was above 95% under these situations and did not alter much after 6 h at room temperature.

Effect of antioxidant agent amount on labeling

Stability of the complex increased slightly in the presence of ascorbic acid, but not significantly. A decrease in the R/H $^{99m}\text{TcO}_4^-$ percentage increases labeling efficiency. Figure 3 was illustrated the results. Since the amount of auxiliary material should always be decreased, ascorbic acid-free samples were chosen for continued study.

Effect of incubation time on labeling

Stannous chlorides (20 μg) including formulations were labeled with 37 MBq $^{99m}\text{TcO}_4^-$. RP of the complexes was studied by RTLC tests done at 5, 15, 30, 45, and 60 min after radiolabeling. According to the trials, the best radiolabeling yield (~98%) was obtained after a 15 min incubation time (Figure 4).

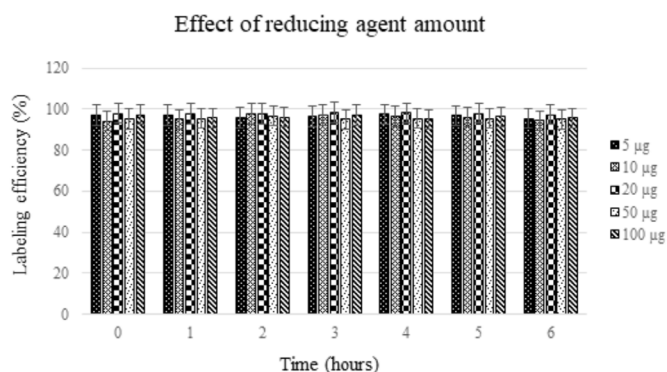


Figure 2. The labeling efficiency of ^{99m}Tc -IBD in which different amounts of stannous chlorides

^{99m}Tc : Technetium-99m, IBD: Ibandronate sodium

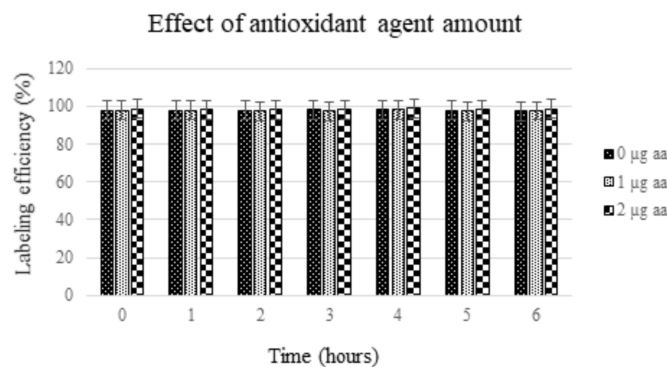


Figure 3. The labeling efficiency of ^{99m}Tc -IBD in the absence and presence of ascorbic acid

^{99m}Tc : Technetium-99m, IBD: Ibandronate sodium

Effect of radioactivity doses on labeling

IBD was radiolabeled with radiation dosages of 37, 185, and 370 MBq $^{99m}\text{TcO}_4^-$. With increasing radioactivity, RP of the ^{99m}Tc]Tc-IBD complex decreased slightly (Figure 5).

Effect of pH on labeling

For pH 4.0 and 7.4, influence of pH on the labeling efficiency of ^{99m}Tc]Tc-IBD was investigated. According to the results of the experiments, pH of the reaction medium plays a significant role in the labeling process (Figure 6). RP was altered, while the other reaction parameters remained constant, and the pH of the reaction was modified from 4.0 to 7.4. The best labeling efficiency was found at pH 5.5, which is the same as IBD's pH.

The effect of filtration on labeling

The labeling efficiency was reduced when ^{99m}Tc]Tc-IBD was filtered. The outcomes are represented in Figure 7.

In vitro stability

Stability of ^{99m}Tc]Tc IBD in saline

The produced radiopharmaceutical's stability was tested at

room temperature for up to 24 h. ^{99m}Tc]Tc-IBD was confirmed to be stable in saline over the test period as indicated by RTLC (Figure 8).

Stability of ^{99m}Tc]Tc-IBD in cell medium

The compound was stable in cell media during the test period as assessed by RTLC. The percentage of ^{99m}Tc]Tc-IBD did not significantly decrease within 2 h as demonstrated in Figure 9.

RTLC procedure

Our research group has developed an innovative, basic, fast, and effective direct technique for ^{99m}Tc labeling of IBD. RTLC tests were used to examine the labeling efficiency of the ^{99m}Tc]Tc-IBD. The quantities of radioactive impurities (free $^{99m}\text{TcO}_4^-$ and R/H $^{99m}\text{TcO}_4^-$) were determined using two solvent systems. RTLC tests were used to evaluate the RP and stability of ^{99m}Tc]Tc-IBD. Free $^{99m}\text{TcO}_4^-$ migrated with the solvent front in RTLC tests using acetone as the solvent, while ^{99m}Tc]Tc-IBD and R/H $^{99m}\text{TcO}_4^-$ remained at the spotting point. R/H $^{99m}\text{TcO}_4^-$ was identified using the mobile phase ACN/W/TFA (50/25/1.5), where R/H $^{99m}\text{TcO}_4^-$ remained at the site of spotting, whereas free $^{99m}\text{TcO}_4^-$ and ^{99m}Tc]Tc-IBD migrated with the solvent front. Figure 10 shows RTLC chromatogram of ^{99m}Tc]Tc-IBD. RP of ^{99m}Tc]Tc-IBD obtained using RTLC was >95%.

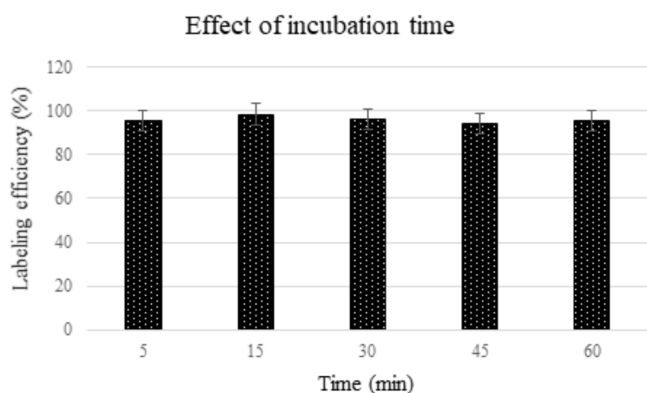


Figure 4. The labeling efficiency of ^{99m}Tc]Tc-IBD at different times post-labeling

^{99m}Tc : Technetium-99m, IBD: Ibandronate sodium

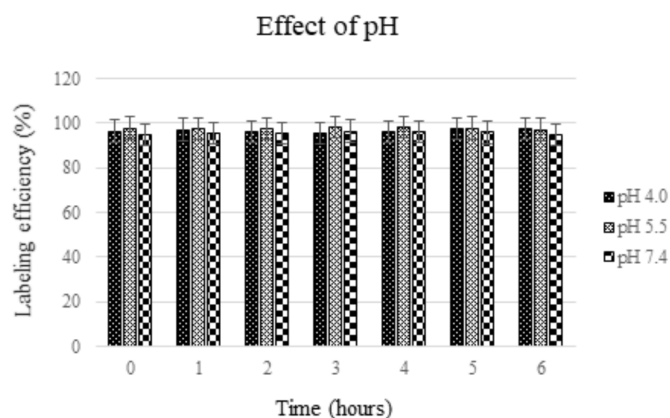


Figure 6. The labeling efficiency of ^{99m}Tc]Tc-IBD on different pH value

^{99m}Tc : Technetium-99m, IBD: Ibandronate sodium

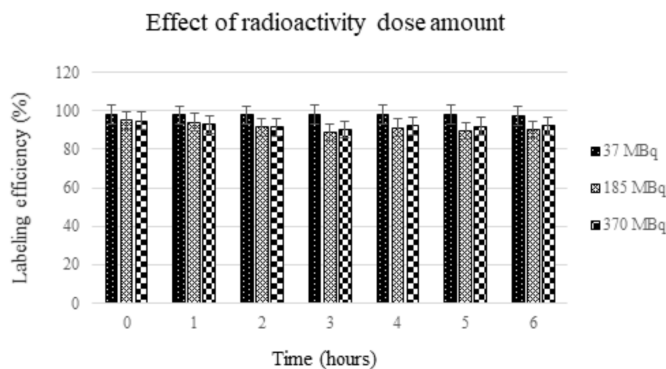


Figure 5. The labeling efficiency of ^{99m}Tc]Tc-IBD in different radioactivity doses

^{99m}Tc : Technetium-99m, IBD: Ibandronate sodium

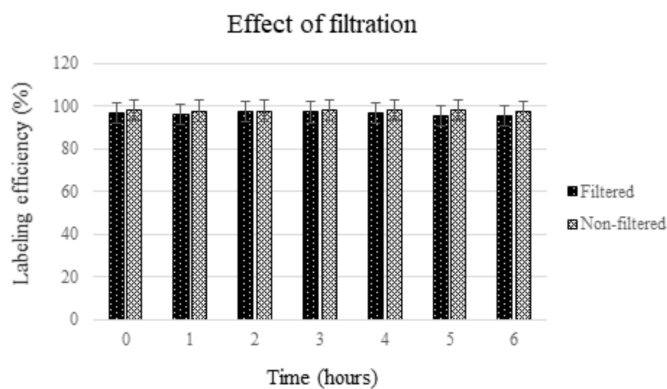


Figure 7. The labeling efficiency of ^{99m}Tc]Tc-IBD in terms of filtration

^{99m}Tc : Technetium-99m, IBD: Ibandronate sodium

In vitro incorporation studies

The percentage of [^{99m}Tc]Tc-IBD and R/H $^{99m}\text{TcO}_4^-$ incorporated into U2OS cell line was investigated in this research. Figure 11 shows the percentage of [^{99m}Tc]Tc-IBD and R/H $^{99m}\text{TcO}_4^-$ incorporated into U2OS cell lines after 30, 60, and 120 min of incubation. During the test period, the maximum incorporation percentage (~80%) was observed with [^{99m}Tc]Tc-IBD up to 2 h. The incorporation percentage of R/H $^{99m}\text{TcO}_4^-$ was decreased during the test period.

Hydroxyapatite-binding studies

The higher binding affinity to bone minerals is responsible for very selective localization and stability of therapeutically used radiolabeled BPs on bone. To determine the bone-seeking properties of [^{99m}Tc]Tc-IBD, *in vitro* HA absorption tests were conducted. In a 5 mg.mL⁻¹ suspension, the percentage of IBD binding to HA was found to be 83.70 ± 3.67 . HA experiment revealed that IBD has a significant capacity for binding to HA than [^{99m}Tc]Tc-MDP (methylene diphosphonate, bone imaging agent in clinical routine), whose binding efficiency was $63.10 \pm 3.0\%$.

Biological tests of [^{99m}Tc]Tc-IBD

Sterility test

Sterility of the IBD solution was demonstrated by the absence of clearly visible microbial growth in the vials at the end of the sterility test.

Isotonicity test

According to British Pharmacopeia, isotonicizing of the IBD solution was found to be 302 mOsm.mL⁻¹, which was suitable for injectable formulation.

Pyrogenicity test

The pyrogenicity test revealed that the IBD solution was non-pyrogenic.

Lipophilicity studies

logP value is a precious measure that, when combined with other factors, may be used to understand a drug's behavior, and predict its distribution throughout the body. logP of the [^{99m}Tc]Tc-IBD was found to be -1.0104. Due of the anionic nature of the produced complexes and the BP groups, the radiolabeled molecule has very polar properties (logP <1).²⁹

DISCUSSION

BPs, such as MDP, ALD, and IBD, have long been used to control cancer-induced bone resorption in cancer patients.³⁰ IBD's bone-seeking feature has been used for cancer imaging and therapy in various ways, including direct conjugation or conjugation to a cargo. The clinical pharmacokinetics of IBD indicate that this BP has powerful bone mineral affinity.³¹

Because of its good physical features and low availability from a generator, ^{99m}Tc has become the most essential nuclide for organ imaging in nuclear medicine. BPs can be labeled with ^{99m}Tc and used for bone imaging because of their good localization in the skeleton and fast clearance from soft tissues. Since Qiu et al.³² discovered that BPs have a high affinity for bone minerals in 1968, several ^{99m}Tc -labeled phosphate compounds have been developed for skeletal imaging.

$^{99m}\text{TcO}_4^-$ was eluted from $^{99}\text{Mo}/^{99m}\text{Tc}$ generator in +7 oxidation level. Since the chemical reactivity of the $^{99m}\text{TcO}_4^-$ anion is so minimal, presence of a suitable reducing agent in the proper ratio is essential for successful direct labeling with ^{99m}Tc . Because to its quick action, perfect redox characteristics, and efficacy at room temperature, stannous chloride is the most often used reducing agent.²⁵ Here, stannous chloride was used as a reducing agent.

In the preparation of [^{99m}Tc]Tc-IBD, three types of ^{99m}Tc could be present: (1) $^{99m}\text{TcO}_4^-$ that has not been reduced by stannous chloride, (2) R/H $^{99m}\text{TcO}_4^-$ that did not react with IBD and was bonded to hydrolyzed stannous chloride, and (3) [^{99m}Tc]Tc-IBD (the desired molecule).

Generally, using an excess of stannous chloride for ^{99m}Tc reduction can result in the desired radiolabeled complexes, but it can also create unwanted radiocolloids that are uptaken by macrophages and so concentrate in RES organs, irradiating them unnecessarily. For this reason, the amount of reducing agent is one of the most important parameters in the radiolabeling procedure of any molecule with ^{99m}Tc . The amount

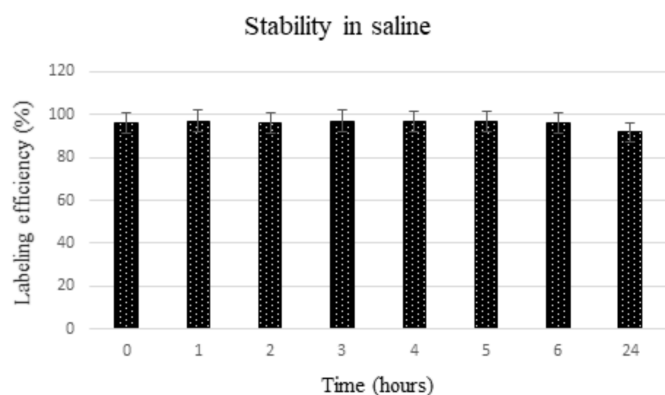


Figure 8. The stability of [^{99m}Tc]Tc-IBD in saline up to 24 h

^{99m}Tc : Technetium-99m, IBD: Ibandronate sodium

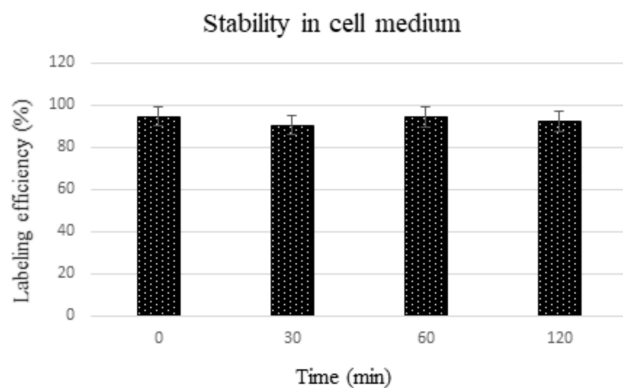


Figure 9. The stability of [^{99m}Tc]Tc-IBD in cell medium up to 2 h

^{99m}Tc : Technetium-99m, IBD: Ibandronate sodium

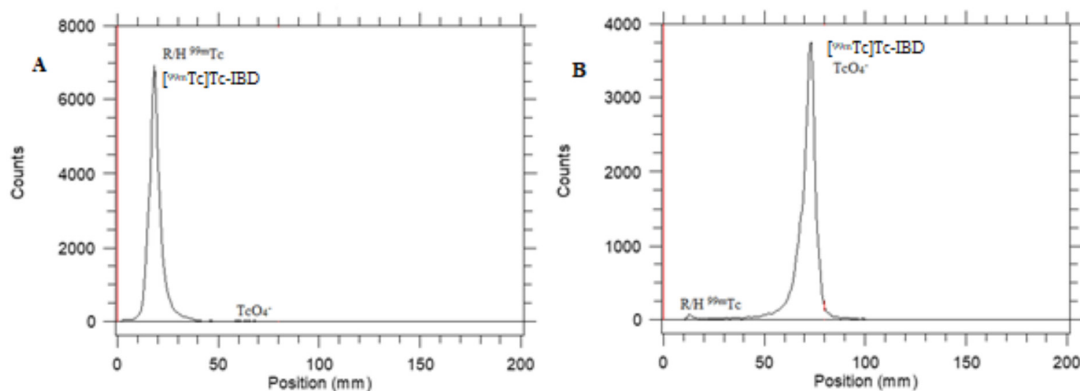


Figure 10. RTLC chromatogram of $[^{99m}\text{Tc}]\text{Tc-IBD}$ in different mobile phases: A: acetone, B: ACN/W/TFA (50/25/1.5)

^{99m}Tc : Technetium-99m, IBD: Ibandronate sodium, RTLC: Radioactive thin layer chromatography, ACN/W/TFA: Acetonitrile/water/trifluoroacetic acid

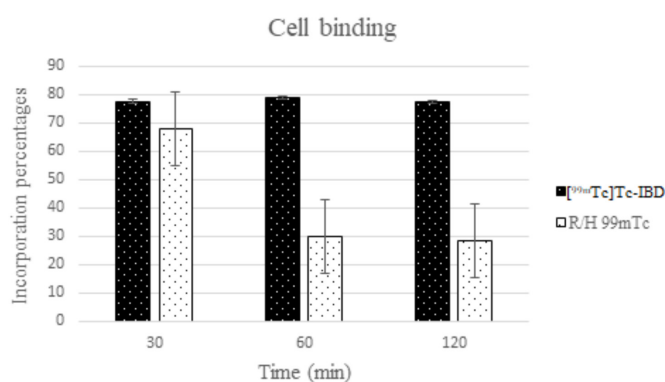


Figure 11. Incorporation percentage of $[^{99m}\text{Tc}]\text{Tc-IBD}$ and R/H ^{99m}Tc to the U2OS cell lines for 30, 60, and 120 min

^{99m}Tc : Technetium-99m, IBD: Ibandronate sodium

of stannous chloride needed for efficient ^{99m}Tc labeling of IBD was found to be 20 μg (Figure 2).

For RTLC studies, ITLC-SG with two mobile phases [acetone and ACN/W/TFA (50/25/1.5)] (Figure 10) was used to study the effects of reaction pH and incubation time, and the amount of the reducing agent on the radiolabeling yield and stability of $[^{99m}\text{Tc}]\text{Tc-IBD}$. IBD was radiolabeled with ^{99m}Tc with high yields (95%) at pH 5.5 (Figure 6). After 15 min at room temperature, radiolabeling was confirmed to be complete (Figure 4). No further purification was required because the radiolabeling yield of $[^{99m}\text{Tc}]\text{Tc-IBD}$ complexes was greater than 95%. Additionally, $[^{99m}\text{Tc}]\text{Tc-IBD}$ was quite stable and labeling efficiency was found >90% for 24 h in SF (Figure 8) and >90% for 2 h in cell medium (Figure 9).

In vitro cell binding tests revealed that $[^{99m}\text{Tc}]\text{Tc-IBD}$ had the highest cell binding capability for U2OS cells, and that binding ratio of $[^{99m}\text{Tc}]\text{Tc-IBD}$ to bone cancer cells was higher than that of R/H $^{99m}\text{TcO}_4^-$. According to the data, the cell binding ratio did not alter at any of the time periods (30, 60 or 120 min) (Figure 11). In addition to this result, the percentage of IBD binding to HA was found to be 83.70 ± 3.67 that has high incorporated to bone minerals.

IBD was previously labeled with ^{99m}Tc and bone uptake was evaluated.³³ According to this study, IBD (300 μg) was radiolabeled with high radiolabeling efficiency (98.6%) using 25 μg of stannous chloride at room temperature at pH 7 for 15 min incubation time. Then, the radiolabeled complex was purified by high performance liquid chromatography (HPLC). Although the *in vitro* stability study was determined for 8 h, $[^{99m}\text{Tc}]\text{Tc-IBD}$ was found to be stable for 6 h. According to the performed biodistribution study, $[^{99m}\text{Tc}]\text{Tc-IBD}$ indicated high uptake and long retention in bone (44.32 % ID/organ) at (1 h) post-injection. Thus, it was concluded that $[^{99m}\text{Tc}]\text{Tc-IBD}$ could be used as a selective potential imaging agent for diagnosis of bone diseases.³³

CONCLUSION

In this study, we demonstrated that IBD can be labeled with ^{99m}Tc with high RP (>95%) using a simple RTLC approach. The resultant complex was extremely stable with labeling efficiency maintaining up to 6 h. At pH 5.5, formulations containing 20 μg stannous chloride and 37 MBq $^{99m}\text{TcO}_4^-$ yielded the highest RP.

According to the cell culture data, $[^{99m}\text{Tc}]\text{Tc-IBD}$ was demonstrated to be a valuable tool for determining the cancer cell binding affinity *in vitro*. Incorporation of $[^{99m}\text{Tc}]\text{Tc-IBD}$ to U2OS cells was greater than that of R/H $^{99m}\text{TcO}_4^-$. Because of these encouraging radiolabeling and cell culture results, $[^{99m}\text{Tc}]\text{Tc-IBD}$ will be studied further in nuclear medicine patients for bone cancer diagnosis.

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Ethics

Ethics Committee Approval: Not applicable.

Informed Consent: Not applicable.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: M.E., D.İ.Ö., Concept: M.E., D.İ.Ö., M.A., Design: M.E., D.İ.Ö., Data Collection or Processing: M.E., D.İ.Ö., E.Ö., Analysis or Interpretation: M.E., D.İ.Ö., E.G., Literature Search: M.E., D.İ.Ö., Writing: M.E.

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