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Original Article

Radiolabeling of Ceftriaxone with ^{99m}Tc as a Targeting Radiopharmaceutical for *Staphylococcus Aureus* Detection in Mouse Model

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Abstract

Introduction
Bacterial infection is one of the major causes of morbidity and mortality especially in developing countries. Nuclear medicine has an important role in helping the diagnosis of deep-seated infections by developing more specific radiopharmaceuticals. The aim of this study was to evaluate 99mTc-labeling ceftriaxone as a new radiopharmaceutical for Staphylococcus aureus infection imaging in nuclear medicine.
Materials and Methods
Radiolabeling of ceftriaxone was carried out by adding 370 MBq of 99mTc to 10 mg of ceftriaxone in the presence of 50 µg of $SnCl_2.2H_2O$ at pH=5. The radiochemical purity and stability tests at room temperature and human blood serum were evaluated with ITLC. Intramuscular infection was induced by injection of Staphylococcus aureus into the left thigh muscle of the mice. The biodistribution of 99mTc-ceftriaxone was studied in normal and infected mice at various times post-injection.
Results
Radiochemical purity of the product was 94.5±5.4% with a good stability at room temperature and human serum, 80.6% and 71.2% after 24 h, respectively. The biodistribution studies showed the localization of 99mTc-ceftriaxone at the site of infection with high sensitivity without any significant accumulation in vital organs.
Conclusion
Due to the ease of 99mTc-ceftriaxone conjugation method, high labeling efficiency, and high uptake in the infected muscle, it may provide a promising candidate as a targeting radiopharmaceutical for imaging infectious foci due to Staphylococcus aureus in nuclear medicine.
Keywords: Ceftriaxone, Infection, Radiopharmaceutical, *Staphylococcus Aureus*, Targeting, 99mTc-radiolabeling,

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1. Introduction

Although our understanding of microorganisms has advanced significantly and antimicrobial therapy has become increasingly available, infection remains a major cause of patient morbidity and mortality [1]. Localizing and distinguishing the "infection" in body sites are very important and life saving processes. The identification of an infection at early stages of the disease is critical for a favorable outcome [2]. Although, imaging techniques such as X-ray, computerized tomography (CT), magnetic resonance imaging (MRI), and ultrasonography might be helpful, but none of these techniques are specific for infection diagnosis because of their limitations due to insignificant anatomical changes in the early stages of the infection process [2,3]. In contrast, nuclear medicine procedures can determine the location and the degree of disease activity in infectious processes based on physiologic and/or metabolic changes that are associated with these diseases [4]. These methods require reliable radiopharmaceuticals that can selectively concentrate in sites of infection [5]. In recent years, the development of radiolabeled antimicrobial agents for specific imaging of infections has received considerable attention [6]. The use of radiolabeled antibiotics is rapidly emerging as a promising diagnostic test for detecting infective lesions, because of their specific binding to the bacterial component [7]. The first antibiotic labeled with ^{99m}Tc was ciprofloxacin, a member of flouroquinolones group (1996-1998) [8]. ^{99m}Tc-labeled ciprofloxacin (Infecton) was introduced as a new class of radiopharmaceuticals for infection imaging [9-11]. Besides, the radiolabeling of some other members of flouroquinolones, cephalosporins, and also some antituberculous agents, has been investigated [8, 12]. Mirshojaei et al. produced radiolabeled ciprofloxacin using ^{99m}Tc and prepared its freeze-dried cold kit as an infection imaging agent in 2010 [13]. Ceftriaxone (Figure 1) is a third generation cephalosporin with a broad spectrum activity against the majority of aerobic and anaerobic gram positive and gram negative pathogenic bacteria. It is used in serious infections of lower respiratory tract, urinary tract, skin, and bone infections and also gonococcal infections, bacteremia, septicemia, and meningitis [14, 15].

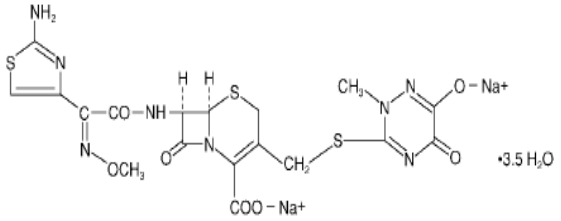

Figure 1. Chemical structure of ceftriaxone sodium.

Radiolabeling ceftriaxone with ^{99m}Tc was performed by Mostafa et al. in 2010 for detecting *E. coli* infection with promising results [16]. In the present study, ceftriaxone was radiolabeled using ^{99m}Tc and the labeling efficiency was optimized. Then, the ability of the new compound in detecting *Staphylococcus aureus* infectious foci was investigated in mouse model. *Staphylococcus aureus* is the cause of some major diseases in human beings such as skin and soft tissue infections, pneumonia, osteomyelitis, and endocarditis [17].

2. Materials and Methods

2.1. Materials

All chemicals were obtained from Merck, Germany. Ceftriaxone sodium was obtained from Daana Pharmaceutical Company, Tabriz, Iran. ^{99m}Tc pertechnetate was supplied by Atomic Energy Organization of Iran. ITLC (Instant Thin Layer Chromatography) sheets impregnated with silica gel were purchased from Merck, Germany. BALB/c mice (8-9 weeks old and 20-25 g weight) were purchased from Razi Vaccine & Serum Research Institute, Karaj, Iran. *Staphylococcus aureus* was prepared from microbial bank of Zanjan Azad University, Iran. N_2-purged bidistilled water was used for preparing all solutions.

2.2. Radiolabeling of Ceftriaxone with ^{99m}Tc

At first, radiolabeling ceftriaxone with ^{99m}Tc was performed with described protocol by Mostafa et al. [16]. Thirty mg of ceftriaxone in 0.5 ml of N_2-purged bidistilled water was dissolved into a sterile glass vial. Then, 50 µg of $SnCl_2.2H_2O$ in 0.01 M HCl, as a reducing agent, was added. The pH of solution was adjusted to 9 using 0.1 M NaOH. The volume of the mixture was adjusted to 1 ml by N_2-purged bidistilled water. Thereafter, 370 MBq of freshly eluted pertechnetate solution was added into the vial. The reaction mixture was incubated at room temperature for 15 minutes. Then, the labeling yield and radiochemical purity of the product was determined.

2.3. Radiochemical Purity of ^{99m}Tc-Ceftriaxone

Radiochemical purity of ^{99m}Tc-ceftriaxone was determined by thin layer chromatography method using ITLC-SG strips. The other metabolites of ^{99m}Tc (free $^{99m}TcO_4^-$ and ^{99m}Tc colloid) were determined by this technique, too. For this purpose, 5 µL of the production was spotted on 2 silica gel-impregnated ITLC strips (1.5×12 cm^2) and developed in acetone and ethanol: water: ammonium hydroxide mixture (2:5:1) as the mobile phase. After complete development, the two radiochromatograms were dried, cut into 1 cm pieces and separately counted using the NaI (Tl) scintillation counter to determine the ratio of the hydrolyzed ^{99m}Tc, free $^{99m}TcO_4^-$, and ^{99m}Tc-ceftriaxone. In acetone system, pertechnetate migrated to the front of the mobile phase (R_f =1.0) and the R_f–values for both ^{99m}Tc-ceftriaxone and ^{99m}Tc colloid were 0. In the mixture of 2:5:1 system, the colloid was found at the origin of the strip (R_f =0) and that of both ^{99m}Tc-ceftriaxone and free $^{99m}TcO_4^-$ were 1. Therefore, by calculating the free $^{99m}TcO_4^-$ and ^{99m}Tc colloid, the labeled ^{99m}Tc-ceftriaxone was well characterized [18, 19].

2.4. Radiolabeling Optimization

Optimization of the affecting factors on radiolabeling of ceftriaxone with ^{99m}Tc was studied using ITLC-SG. The amounts of 2-60 mg of ceftriaxone for determining the best concentration for high labeling yield were used. The optimum pH for the labeling of ^{99m}Tc-ceftriaxone was adjusted using HCl or NaOH at various pH values (3-7). To determine the optimum amount of reducing agent, 20-100 µg of $SnCl_2.2H_2O$ in 0.01 M HCl was used. For determination of the appropriate reaction time, the vial was incubated at various intervals at room temperature to obtain the stability and the highest labeling yield of the labeled product. The reaction mixture was heated in a 60 ºC water bath for 10 minutes to find the effect of heating on the labeling efficiency.

2.5. ^{99m}Tc-ceftriaxone Stability at Room Temperature and Human Blood Serum

The stability of the ^{99m}Tc-ceftriaxone was studied at room temperature and human blood serum using ITLC-SG. For this purpose, by incubating the labeled product at room temperature at interval times of 10, 20, 30, and 45 minutes and 1, 2, 3, 4, 8, 16, and 24 hours, the best time for obtaining the highest labeling yield was achieved. The complex stability was determined in human serum by adding 200 µl of ^{99m}Tc-ceftriaxone to 1.8 ml human serum at 37 ºC incubator. Then, the serum samples were analyzed at 30 minutes and 1, 2, 3, and after 24 hours.

2.6. Biodistribution Study of ^{99m}Tc-Ceftriaxone in Normal BALB/c mice

The biodistribution pattern of ^{99m}Tc-ceftriaxone in 8 groups of normal BALB/c mice (n=5) was performed at 15, 30, and 60 minutes and 4, 6, 8, 16, and 24 hours after injection of 100 µL of radiolabeled compound via the tail vein. The mice were sacrificed at each time points by suffocation using CO_2 gas. Then, tissue organs such as heart, blood, kidneys, liver, spleen, stomach, intestines, lungs, and muscles were dissected and removed. The organs were weighed and the ^{99m}Tc activity was counted using the gamma counter. Thereafter, the average percentage of

radioisotopes injected dose per gram of tissue (%ID/g) was calculated.

2.7. Biodistribution Study of ^{99m}Tc-Ceftriaxone in Infected BALB/c mice

Overnight cultures of *Staphylococcus aureus* were prepared on nutrient agar plates at 37 ºC incubator. A turbid suspension containing 1×10^8 CFU of viable *bacteria* in 0.2 ml of saline was injected intramuscularly to the left lateral thigh muscle of 20 BALB/c. Twenty four hours were required to get gross swelling in the infected thighs. Biodistribution study of ^{99m}Tc-ceftriaxone was performed in 4 groups of infected BALB/c mice (n=5) at 30 minutes and 1, 2, and 4 hours after injection of ^{99m}Tc-ceftriaxone. One hundred µL of radiolabeled compound was intravenously injected via the tail vein. After sacrificing the animals using CO_2 gas, 1 ml of blood was collected from the heart. In addition, different organs such as heart, kidneys, liver, spleen, stomach, intestines, lungs, and right and left thigh muscles (normal/infected thigh muscles) were removed and quickly washed with saline and then weighed. The distribution of radioactivity in organs and thighs was determined by well-type gamma counter. The results were expressed as the average percentage of injected dose per gram of tissue (% ID/g).

2.8. Statistical Analysis

All the experiments related to radiolabeling procedure, optimization, and quality control, were repeated five times and the results were expressed as mean±SD. ANOVA test was used to compare the distribution data of radiolabeled product at infected and normal muscles ($p < 0.05$).

3. Results

3.1. Radiochemical Purity

Radiolabeling of ceftriaxone with ^{99m}Tc at reaction conditions of 30 mg ceftriaxone, 50 µL of $SnCl_2.2H_2O$, pH=9, and 15 minutes incubation time at room temperature, resulted in 83.6±3.4 % radiolabeling yield.

3.2. Radiolabeling Optimization

3.2.1. Ceftriaxone

The results of using 2-60 mg of ceftriaxone (reaction conditions: pH=9, 50 µL $SnCl_2.2H_2O$, and 15 minutes incubation time at room temperature) showed that by increasing the ceftriaxone amount, the labeling yield increases and after 10 mg the changes were not significant.

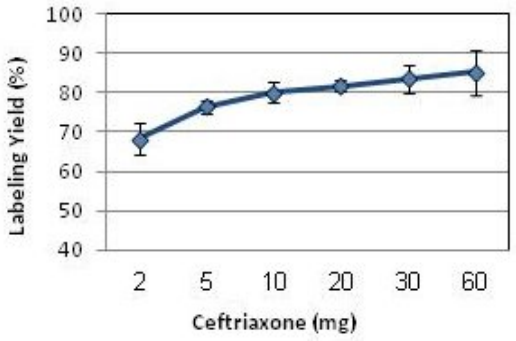

Figure 2. The effect of ceftriaxone amount (2-60 mg) on the labeling yield of ^{99m}Tc-ceftriaxone (n=5).

3.2.2. pH

In optimization of pH values (reaction conditions: 10 mg ceftriaxone, 50 µL $SnCl_2.2H_2O$, and 15 minutes incubation time at room temperature), the maximum labeling yield (91.8±2.4%) was obtained at pH=5. Hence, next experiments were performed at pH=5 (Figure 3).

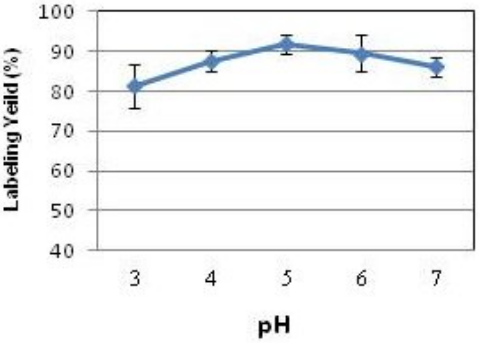

Figure 3. The effect of pH (3-7) on the labeling yield of ^{99m}Tc-ceftriaxone (n=5).

3.2.3. $SnCl_2.2H_2O$

The effect of various amounts of reducing agent on labeling yield (reaction conditions: 10 mg ceftriaxone, pH=5, and 15 minutes incubation time at room temperature) showed that by using of 50 µL $SnCl_2.2H_2O$ in 0.01 M HCl, the highest labeling yield was about 91.8±2.4% (Figure 4).

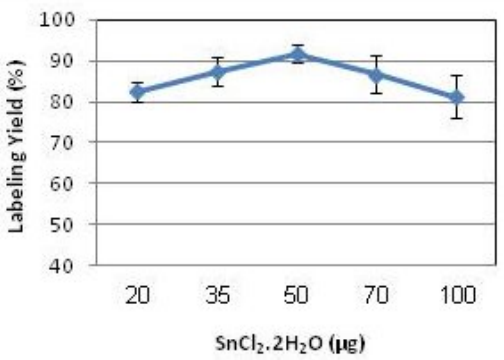

Figure 4. The effect of $SnCl_2.2H_2O$ (20-100 µg) on the labeling yield of ^{99m}Tc-ceftriaxone (n=5).

3.2.4. Incubation Time and Reaction Temperature

At reaction conditions of 10 mg ceftriaxone, 50 µL $SnCl_2.2H_2O$, and pH=5, by increasing incubation time at room temperature from 15 minutes to 30 minutes, the labeling yield increased up to 94.5±5.4%. The resulted labeling yield during the heating in a 60 ºC water bath didn't have any significant differences with the obtained yield of 30 minutes incubation time at room temperature and achieved about 93.6%. The amounts of radiocolloids at optimal condition were less than 4%.

3.3. The stability of ^{99m}Tc- Ceftriaxone at Room Temperature and Human Blood Serum

The results showed that the highest labeling yield (94.5±5.4%) was achieved at 30 minutes after starting the reaction and did not decrease to less than 90% up to 3 hours post radiolabeling. The stability of labeled product after 24 hours declined to 80.6±7.2% (Figure 5).

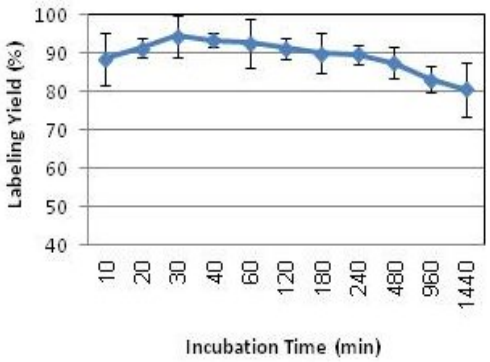

Figure 5. The stability of ^{99m}Tc-ceftriaxone at room temperature up to 24 hours (n=5).

The results of the stability test of ^{99m}Tc-ceftriaxone in human blood serum showed that there was about less than 5% decrease in the labeling efficiency up to 3 hours which was 71.2% stable after 24 hours (Figure 6).

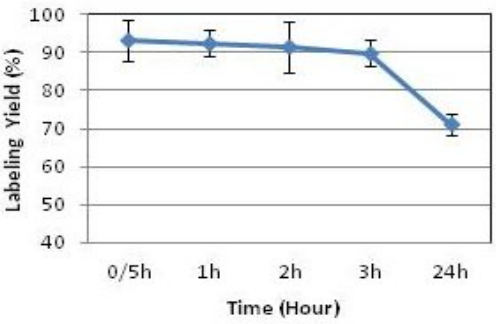

Figure 6. The stability of ^{99m}Tc-ceftriaxone in human blood serum up to 24 hours (n=5).

3.4. Biodistribution Study of ^{99m}Tc-Ceftriaxone in Normal BALB/c mice

The biodistribution results of ^{99m}Tc-ceftriaxone in normal BALB/c mice have been shown in Figure 7. The data showed that there was no unnatural accumulation of labeled product at vital organs. The results showed that at time points after 4 hours post-injection, the percentage of radioactivity in different organs was extremely decreased. Therefore, this time points of biodistribution study were omitted in infected BALB/c mice.

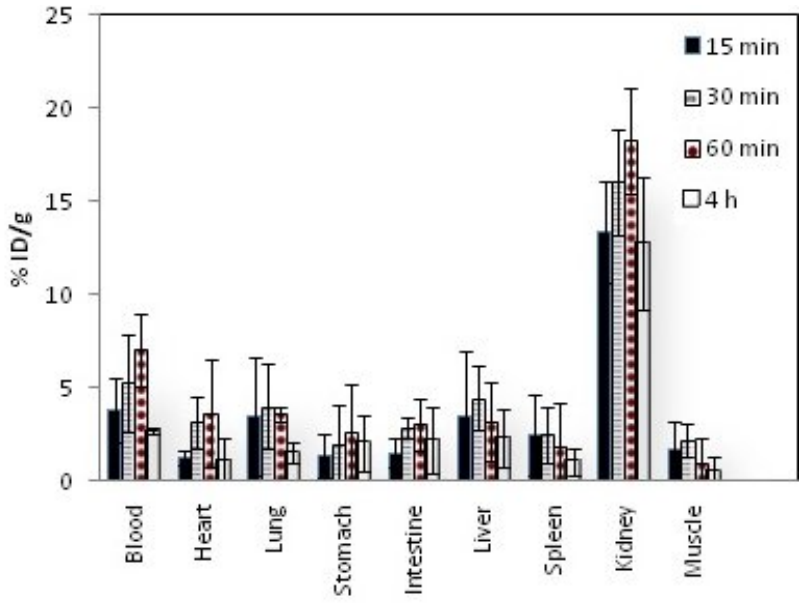

Figure 7. Organ biodistribution of ^{99m}Tc-ceftriaxone in normal BALB/c mice (n=5).

3.5. Biodistribution Study of ^{99m}Tc-Ceftriaxone in Infected BALB/c mice

The resulted data of ^{99m}Tc-ceftriaxone biodistribution in infected BALB/c mice showed that after injection of 100 µL of labeled product via the tail vein of infected mice, the labeled ceftriaxone rapidly distributed in the body organs of the mice. The results also revealed that the labeled product accumulates significantly at the infected thigh muscle in comparison with the normal muscle ($p<0.05$) at 4 hours post-injection (Figure 8).

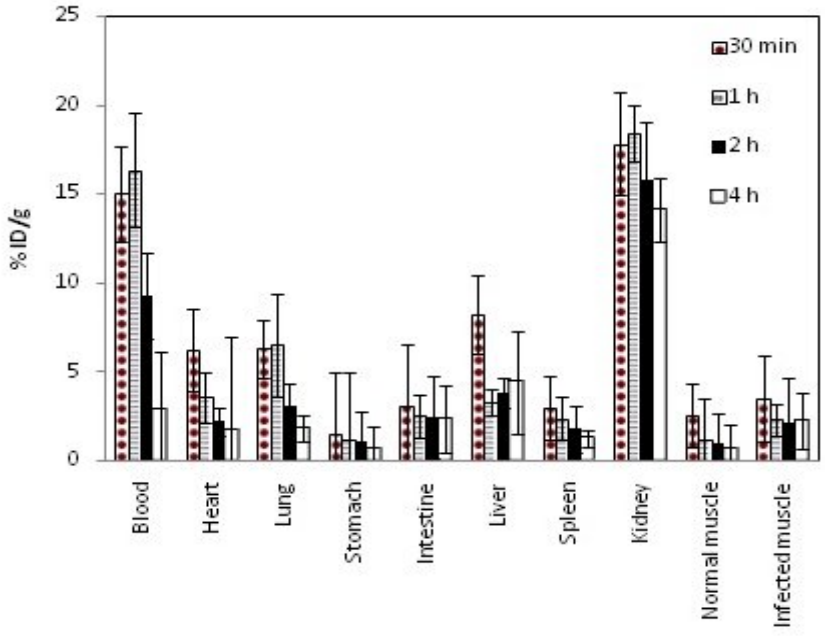

Figure 8. Organ biodistribution of ^{99m}Tc-ceftriaxone in S. *aureus* infected BALB/c mice (n=5).

4. Discussion

In recent years, the use of targeting radiopharmaceuticals as a promising approach for early detection of infections has been developed. In this respect, radiolabeled antibiotics as intelligent tracers can accumulate at the site of infection by direct

targeting of microorganisms. In this study, at first, the radiolabeling of ceftriaxone with ^{99m}Tc was performed according to the presented process by Mostafa et al. that resulted 83.6±3.4% radiolabeling yield [16]. Then, for determining the optimal conditions to gain the highest labeling yield with the maximum stability, the reaction was repeated at different ranges of pH and various amounts of ceftriaxone and $SnCl_2.2H_2O$. The results of optimization of affecting factors on radiolabeling showed that at reaction condition of 10 mg ceftriaxone, 50 µL $SnCl_2.2H_2O$, pH=5, and 30 minutes incubation time, the labeling yield of ceftriaxone reached 94.5±5.4%. The results of the stability tests of ^{99m}Tc-ceftriaxone at room temperature and human blood serum showed that after 3 hours of starting the reaction, the stability decreases about 3-4% and after 24 hours reaches 80.6% and 71.2% at room temperature and human blood serum, respectively. Therefore, it was confirmed that the radiolabeled product has a good stability. It is obvious that, the method of radiolabeling of ceftriaxone is simple, rapid, and efficient with high labeling yield that does not require post-labeled purification. The results of biodistribution studies of ^{99m}Tc-ceftriaxone in normal/infected BALB/c mice revealed that there is no subnormal accumulation of labeled product in vital organs and ^{99m}Tc-ceftriaxone can accumulate at infected tissue with high sensitivity. Since, the main excretion pathway of ceftriaxone is via the kidneys (it excretes via the bile too) [14], the complex accumulation in kidneys was high at longer times that was in accordance with its predominant renal clearance. The biodistribution results showed that the uptake of radioactivity in infected thigh muscle at the intervals of 30 minutes and 1, 2, and 4 hours post-injection were 3.51±2.4, 2.30±0.9, 2.09±2.6, and 2.23±1.6, respectively, and those of normal muscles were 2.56±1.8, 1.17±2.3, 0.89±1.7, and 0.69±1.3, respectively. According to these data, the highest ratio of radioactivity uptake in infected to normal muscle (3.24±1.2) was achieved at 4 hours post-intravenous injection of ^{99m}Tc-ceftriaxone (Table 1).

Table 1. Target to non-target ratio at 30 minutes and 1, 2, and 4 hours post-injection of ^{99m}Tc-ceftriaxone.

	30 min	1-h	2-h	4-h
Activity uptake in normal muscle (%) (Target)	2.4±3.51	0.9±2.30	2.6±2.09	1.6±2.23
Activity uptake in infected muscle (%) (Non Target)	1.8±2.56	2.3±1.17	1.7±0.89	1.3±0.69
Target to Non Target ratio (T/NT)	1.3±1.38	0.3±1.97	1.5±2.34	1.2±3.24

The results showed that radiolabeled ceftriaxone has more affinity to adhere to *S. aureus* infected muscle in comparison with the normal muscle ($p<0.05$) that demonstrates the targeting manner of the radiolabeled complex. At 6 and 8 hours post-injection, the percentage of radioactivity in infected and normal muscle was extremely decreased, so the data was omitted for summarizing.

5. Conclusion

Our results are evident that ^{99m}Tc-ceftriaxone complex was accumulated at *Staphylococcus aureus* infectious site with high sensitivity. The application of the new radiopharmaceuticals for detecting infectious foci in human beings needs further investigation that is the aim of authors in the future. This is the first time that radiolabeling of ceftriaxone with ^{99m}Tc is performed for targeting detection of *Staphylococcus aureus* infection in mouse model.

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References

1. Love C, Palestro CJ. Radionuclide imaging of infection. J Nucl Med Technol. 2004;32(2):47-57.
2. Yurt Lambresht F, Yilmaz O, Unak P, Seyitoglo B, Durkan K, Baskan H. Evaloation of 99mTc- cefuroxime axetil for imaging of inflammation. J radioanalytical Nucl Chem. 2008;277(2):491-4.
3. El-Ghany EA, El-Kolaly T, Amine AM, El-Sayed AS, Abdel-Gelil F. Synthesis of 99mTc–pefloxacin: a new targeting agent for infectious foci. J radioanalytical Nucl Chemistry. 2005;266(1):131-9.
4. Gemmel F, Dumarey N, Welling M. Future diagnostic agents. Semin Nucl Med. 2009;39(1):11-26.
5. Agency IAE. Development of Kits for 99mtc Radiopharmaceuticals for Infection Imaging: International Atomic Energy Agency; 2004.
6. Oyen WJ, Corstens FH, Boerman OC. Discriminating infection from steril inflammation. Can radiolabelled antibiotics solve the problem? Eur J Nucl Med Mol Imaging. 2005;32(2):151-2.
7. Singh B, Mittal BR, Bhattagharya A, Aggarwal A, Nagi ON, Singh AK. Technetium-99m ciprofloxacin imaging in the diagnosis of postsurgical bony infection and evaluation of the response to antibiotic theraoy: A case report. J Orthop Surg (Hong Kong). 2005;13(2):190-4.
8. Benitez A, Roca M, Martin-comin J. Labeling of antibiotics for infection diagnosis. Q J Nucl Med Mol Imaging. 2006;50(2):147-52.
9. Vinjamuri S, Hall AV, Solanki KK, Bomanji J, Siraj Q, O'Shaughnessy E, Das SS, Britton KE. Comparison of 99mTc Infecton imaging with radiolabelled white-cell imaging in the evaluation of bacterial infection. Lancet;1996;347(8996):233-5.
10. Hall AV, Solanki KK, Vinjamuri S, Britton KE, Das SS. Evaluation of the efficacy of 99mTc-Infecton, a novel agent for detecting sites of infection. J Clin Pathol. 1998;51(3):215-9.
11. Britton KE, Vinjamuri S, Hall AV, Solanki K, Siraj QH, Bomanji J, Das SS. Clinical evaluation of technetium-99m infecton for the localization of bacterial infection. Eur J Nucl Med. 1997;24(5):553-6.
12. Lambrecht FY. Evaloation of 99mTc-labeled antibiotics for infection detection. Ann Nucl Med. 2011;25(1):1-6.
13. Mirshojaei SF, Erfani M, Sadat Ebrahimi SE, Talebi MH. Freeze- Dried cold kit for preparation of 99mTc-ciprofloxacin as an infection imaging agent. Iran J Nucl Med. 2010;18(2):46-51.
14. Brunton L, Blumenthal D, Buxton I, Parker K. Goodman and Gilman's Manual of Pharmacology and Therapeutics: McGraw-Hill; 2007.
15. Scheld WM. The potential uses of ceftriaxone. Eur J Clin Microbiol. 1983;2(5):485-8.
16. Mostafa M, Motaleb MA, Sakr TM. Labelling of ceftriaxone for infective inflammation imaging using 99mTc eluted from 99Mo/99mTc generator based on zirconium molybdate. Appl Radiat Isot. 2010;68(10):1959-63.
17. Bartlett AH, Hulten KG. Staphylococcus aureus pathogenesis: secretion systems, adhesins, and invasins. Pediatr Infect Dis J. 2010;29(9):860-1.
18. Chattopadhyay S, Saha Das S, Chandra S, De K, Mishra M, Ranjan Sarkar B, et al. Synthesis and evaluation of 99mTc-moxifloxacin, a potential specific imaging agent. Appl Radiat Isot. 2010;68(2):314-6.
19. Siaens RH, Rennen HJ, Boerman OC, Dierckx R, Slegers G. Synthesis and comparison of 99mTc-enrofloxacin and 99mTc-ciprofloxacin. J Nucl Med. 2004;45(12):2088-94.