Optimization condition in labeling of Ofloxacin with ^{99m}Tc and its biological evaluation in Staphylococcus aureus and Escherichia coli for infection imaging

Original Article

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ABSTRACT

Introduction: The use of radiopharmaceuticals is a powerful tool in the management of patients with infectious or inflammatory diseases in nuclear medicine. In this study ofloxacin as a second-generation fluoroquinolone is used to design a desired infection imaging agent after labeling with ^{99m}Tc via direct labeling.

Methods: Ofloxacin was radiolabeled with ^{99m}Tc using different concentrations of ligand, stannous chloride, sodium pertechnetate and at different pH. Then labeling yield, stability in saline and serum, lipophilicity, binding with Staphylococcus aureus and Escherichia coli and biodistribution in infected mice for labeled compound were studied.

Results: The final complex was characterized by TLC and HPLC and radiochemical purity of >90% was obtained when 1.5 mg ofloxacin in presence of 75 µg $SnCl_2$ was labeled with 370 MBq sodium pertechnetate. The complex showed specific binding to Staphylococcus aureus and Escherichia coli. Biodistribution results showed that radioligand had high affinity in the infected site in mice. The uptake for Staphylococcus aureus induced infections (T/NT = 2.33 ± 0.17 at 1 h post injection) was higher than that was for Escherichia coli (T/NT = 1.96 ± 0.13 at 1 h post injection).

Conclusion: This complex may lead to further development of a radiotracer for imaging of infections induced by gram-positive or gram-negative bacteria.

Key words: Ofloxacin; Infection; ^{99m}Tc; Staphylococcus aureus; Escherichia coli; Direct labeling

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INTRODUCTION

The early diagnosis of infection from sterile inflammation is one of the most common challenges in nuclear medicine and for this reason several radiopharmaceuticals have been developed to find the solution for this problem. ^{67}Ga-citrate is one of the early radionuclides for this purpose, but unfortunately with disadvantages like long physical half-life, high and multiple energy gamma rays causing high radiation absorbed doses, non-specificity in infectious or non-infectious inflammations and last but not least not being available as a generator [1]. Leukocytes labeled with ^{99m}Tc or ^{111}In have been considered as a gold standard for the scintigraphy of infection in nuclear medicine [2]. Labeling the leukocyte, it is technically difficult and time-consuming with potential risk of contamination and transmission of blood-borne pathogens to the patient or technologist. This technique is time and potential of or is high [3]. Three phase bone scanning method is highly sensitive tools for discrimination between soft tissues vs. bone infection particularly when uptake on all three phase is positive but it unfortunately is not specific [4]. Antimicrobial peptides, produced by phagocytes, epithelial cells, endothelial or many other cell types, show antibacterial, antiviral, and antifungal activities in vitro [5]. In several previous studies, the ^{99m}Tc labeled cationic antimicrobial peptide derived from human ubiquicidine (UBI) was introduced for detection of bacterial and fungal infections [6-9].

The broad spectrum antibiotic agents have been suggested as promising diagnostic tools for the detection of infection lesions. The antibiotic molecules accumulate at the site of infection due to their metabolism by microorganisms [10]. The majority of the antibiotics studied in this field are those of the quinolones family, second and third generation cephalosporins [11-13].

Recently ^{99m}Tc-Ciprofloxacin has been developed as a new radiopharmaceutical for preferentially diagnosing infection from sterile inflammation [14]. Solanki et al. labeled ciprofloxacin with ^{99m}Tc in 1993, supplied under the name of Infecton [15]. They used two-vial kits for final preparation, whereas most of the clinically used radiopharmaceuticals in nuclear medicine imaging are single-vial kits. Besides, significant amount of colloid which forms upon reconstitution with ^{99m}Tc has also been reported with this kit [16].

Ofloxacin is a broad-spectrum fluroquinolone antibiotic that is active against both gram-positive and gram-negative bacteria. To utilize the enhanced potency of the ofloxacin for localization of infection caused by Staphylococcus aureus and Ecsherichia Coli in the current investigation, radiolabeling of the ofloxacin with ^{99m}Tc was evaluated. Optimization of labeling condition, stability in serum albumin, lipophilicity, binding with Staphylococcus aureus and Escherichia coli and biodistribution in infected mice were studied.

METHODS

Ofloxacin was purchased from Exir pharmaceutical company. Other chemicals were purchased from Merck and Fluka and they were used without further purification. Technetium-99m as sodium pertechnetate ($Na^{99m}TcO_4$) was obtained from an in-house ^{99}Mo/^{99m}Tc generator using 0.9% saline. Monitoring of all reactions was performed with analytical reverse-phase high performance liquid chromatography (RP-HPLC) on a JASCO 880-PU intelligent pump HPLC system (Tokyo, Japan) equipped with a multiwavelength detector and a flow-through Raytest-Gabi γ-detector. CC 250/4.6 Nucleosil 120-5 C-18 column from Teknokroma was used for HPLC. Radioactivity measurements were carried out using Na (Tl) scintillation counter (ORTEC Model 4001 M Minibin & Power Supply).

Radiolabeling

200 mg ofloxacin was dissolved in 10 mL distilled water and 25-100 µl of this solution was carefully transferred to a vial. To this solution 40-100 µg $SnCl_2$ (20-50 µL of 2 mg/mL $SnCl_2$, $2H_2O$ in nitrogen-purged 0.1 M HCl) was added. Finally in different pH ranges 2-8, $^{99m}TcO_4^-$ (185-555 MBq) in 0.5 mL saline was added to the solution and incubated for 30 min at room temperature.

Radiochemical analysis

Radiochemical purity of the ^{99m}Tc-Ofloxacin was analyzed by TLC and HPLC. For the characterization of ^{99m}Tc-ligand, TLC plates were developed in ethanol: water: ammonium hydroxide (2: 5: 1 v/v) as well as in acetone. In the first solvent, free $^{99m}TcO_4^-$ and ^{99m}Tc-ligand move with solvent front with R_f = 0.8-1 and the reduced technetium remain at the point of application. In the second solvent, $^{99m}TcO_4^-$ move with solvent front with R_f = 1 and the other species remain at the point of application. The radioactivity was quantified by cutting the strip (1.5 × 10 cm^2) into 1 cm pieces and counting in a well type gamma counter.

For radiochemical analysis of ^{99m}Tc-ofloxacin by HPLC a volume of 10 µL of the test solutions were injected into the C-18 reverse phase column and 0.1% trifluoroacetic acid/water (Solvent A) and acetonitrile (Solvent B) were used as a mobile phase in the following gradient: 0 min 95% A (5% B), 5

min 95% A (5% B), 25 min 0% A (100% B), 30 min 0% A (100% B), flow = 1 mL/min, λ = 280 nm.

Stability

To one mL of freshly prepared human serum, we added 200 µl (74 MBq) of radiotracer and mixture was incubated in 37 ºC environment. At different time points, 50 µL aliquots was removed and treated with 500 µL of alcohol. Sample was centrifuged for 15 min at 500 rpm to precipitate serum proteins. Supernatant was removed and analyzed with TLC to determine the stability of labeled compound.

Partition coefficient

The octanol/water partition coefficient of complex was measured following 1 min vigorous vortex mixing of 1 ml of octanol and 0.9 ml water, with approximately 50 µl (18.5 MBq) of radiotracer in a micro centrifuge tube. The tubes were centrifuged at 1500 rpm for 2 min and the counts in 100 µl aliquots of both organic and inorganic layers were determined by use of a NaI well-type γ-counter. The reported octanol/water partition coefficient represents the mean (± standard deviation) of the three measurements.

Binding of ^{99m}Tc-Ofloxacin to bacteria

100 µL (37 MBq) of the ^{99m}Tc-ofloxacin was transferred to a test tube. Then, 0.9 mL of 50% (v/v) 0.01 M acetic acid in phosphate buffer (pH = 7.5) containing approximately 1×10^8 colony forming units (CFU) per ml viable S. aureus or E.coli was added. The mixture was incubated for 1 h at 4 ºC and thereafter the vials were centrifuged in a pre-cooled centrifuge for 5 min at 2000 g at 4 ºC. The supernatant was removed, and the bacterial pellet, was gently re-suspended in 1 mL of buffer and re-centrifuged as above. The supernatant was removed and the radioactivity in the bacterial pellet was determined by a gamma counter. The radioactivity related to bacteria was expressed in percent of the added ^{99m}Tc activity bounded to viable bacteria in regard to total ^{99m}Tc.

Animal biodistribution

Male mice, weighing 25-30 g were infected by injection 0.1 mL of saline containing 1×10^8 CFU bacteria into right tight muscle. After 24 h, they were injected under ether anesthesia with 100 µL (37 MBq) solution of ^{99m}Tc-ofloxacin in saline via the tail vein. At 1, 4 and 4 h after injection, accumulation of the tracer in infected area was assessed by planar scintigraphy under ether anesthesia. For ex vivo counting, the mice were sacrificed after 1, 4 and 24 h and the organs of interest were collected, weighed and radioactivity was measured in a gamma counter.

RESULTS AND DISCUSSION

Various complexes of ^{99m}Tc may be formed by interactions between electron donor atoms such as nitrogen, oxygen, sulfur and reduced technetium [17]. Due to presence of electron donor atoms in ofloxacin structure the reduced sodium pertechnetate can easily react with this ligand and a complex is formed (Figure 1). Although the exact complex structure is not known, the proposed structure of the bidentate radiocomplex will have a square planner pyramidal geometry with ^{99m}Tc=O bond in apical while four oxygen atoms from two ofloxacin ligand cover four remaining positions in the base surface. To determine exact structure of the complex further research is necessary.

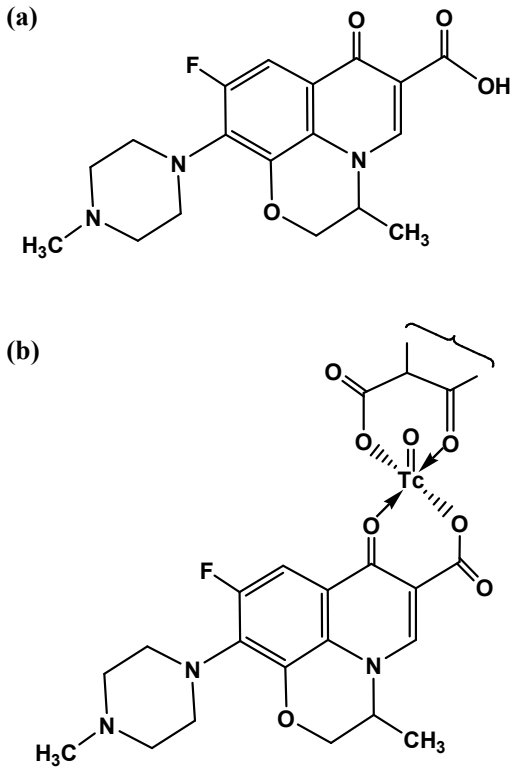

Fig 1. Chemical structure of the Ofloxacin (a). Proposed structure of ^{99m}Tc-Ofloxacin complex (b).

Ofloxacin was labeled with ^{99m}Tc using different concentration of ligand, reducing agent and sodium pertechnetate at different pH. Optimization studies for acquiring maximum complexation yield showed that using 1.5 mg ofloxacin as a bidentate ligand, 75 µg stannous chloride dihydrate as reducing agent,

370 MBq sodium pertechnetate as a radiometal at a pH = 4 higher yield of radioligand was obtained. Effect of different concentration of $SnCl_2$ and different pH range in yield of labeling are showed in Figures 2 and 3, respectively.

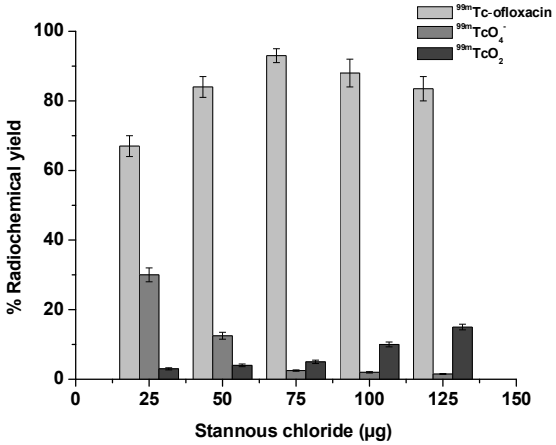

Fig 2. Effect of amount of stanous chloride as a reducing agent on radiochemical purity values of the ^{99m}Tc-Ofloxacin.

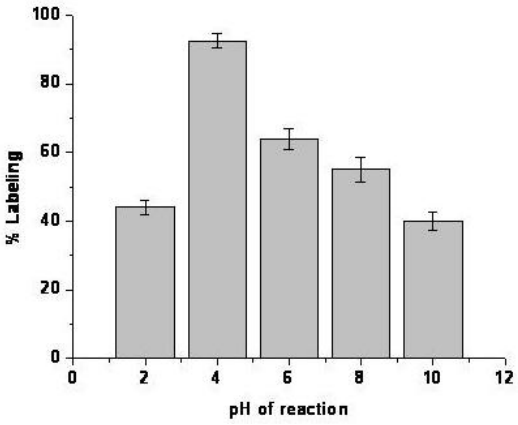

Fig 3. Effect of pH on labeling yield of ^{99m}Tc-ofloxacin.

As results in Figure 2 shows higher yield was achieved by using 75 µg of $SnCl_2$ as a reducing agent. Lower than this value, the reducing agent is not sufficient for complete reduction of pertechnetate for formation of ^{99m}Tc-complex. Increasing the amount above this value also causes decrease in labeling yield. This may be due to the fact that the in the presence of excess reducing agent technetium could rapidly reduces to lower oxidation state producing insoluble technetium form, $^{99m}TcO_2$. These results also show that reaction was favorable at acidic pH leading to >90% complex formation but moving toward higher pH values lowers the yield.

In the radiochemical purity determination of ^{99m}Tc-ofloxacin by TLC, in the first part a solvent system which consisted of ethanol: water: ammonium hydroxide (2: 5: 1 v/v) only minimal activity (less than 6%) remained in origin corresponding to reduced technetium-99m. In the second part choosing acetone as a solvent, majority of activity remained in the origin and less than 3% of total activity was moved and counted in R_f = 1 which belonged to $^{99m}TcO_4^-$. ^{99m}Tc-ligand HPLC studies also demonstrated that the reaction was lead to a single complex and its retention time was found to be 13.48 min which was found to coincide with the UV signal with a yield of more than 90% (Figure 4).

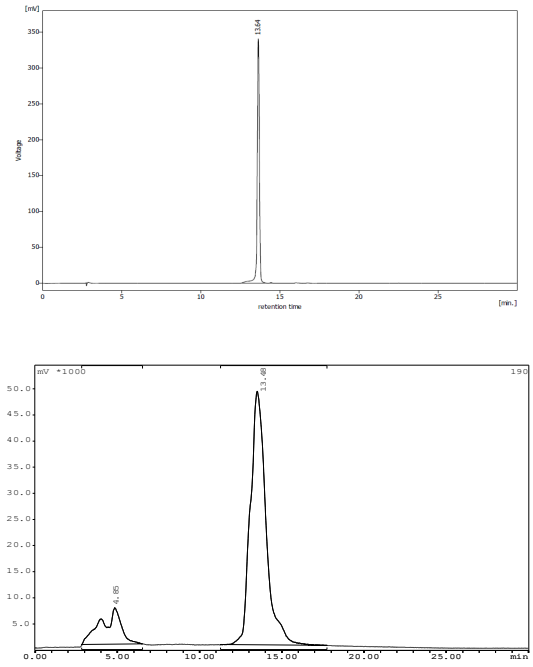

Fig 4. RP-HPLC profile of (above) unlabeled Ofloxacin with UV detector and γ = 280 nm (below) ^{99m}Tc-Ofloxacin complex.

The radiochemical purity of the ^{99m}Tc-ofloxacin was nearly constant (>90%) over the observed period of 6 h. No decomposition of the complex was observed in this time period, suggesting its high stability in the reaction mixtures at room temperature. The affinity of the labeled compound to human serum proteins was about 30 ± 5 % after 6 h. Also labeled antibiotic was stable in human serum with radiochemical purity of more than 85 % after 6 h. So far the main drawback in labeling of ciprofloxacin and its similar structures are colloid impurity and instability which were subject of discussion in previous studies by different groups [12, 14, 18-20] but here with optimization of labeling condition (1.5 mg versus 2 mg ligand, 75 µg versus 50 µg reducing agent and pH = 4 versus 5) high labeling yield and stability for ^{99m}Tc-ofloxacin is achieved.

Table 1. Biodistribution of ^{99m}Tc-ofloxacin in mice (%ID/g ± SD, n=3).

Organ	Time post injection		
	1h	4h	24h
Blood	7.79 ± 0.34	3.47 ± 0.28	0.39 ± 0.05
Kidney	16.47 ± 1.40	10.59 ± 1.21	0.45 ± 0.12
Spleen	3.75 ± 0.47	3.78 ± 0.45	0.60 ± 0.11
Stomach	1.37 ± 0.31	1.32 ± 0.02	0.05 ± 0.01
Intestine	3.14 ± 0.39	5.65 ± 0.49	0.10 ± 0.03
Liver	17.57 ± 1.69	17.19 ± 1.24	1.39 ± 0.21
Lung	4.43 ± 1.00	2.66 ± 0.83	0.33 ± 0.06
Heart	3.55 ± 1.08	1.58 ± 0.86	0.40 ± 0.05
Non-infected muscle	0.84 ± 0.12	0.81 ± 0.10	0.09 ± 0.01
S. aureus infection	1.96 ± 0.22	1.51 ± 0.11	0.17 ± 0.03
E.coli infection	1.65 ± 0.14	1.20 ± 0.10	0.15 ± 0.02

The partition coefficient of the radiolabeled complex was determined by distribution in octanol and water, and the lipophilicity (log P) of ^{99m}Tc-ofloxacin was found to be 1.21 ± 0.11, signifying its moderate lipophilicity which could explain the accumulation of the radioligand in the liver. In vitro binding of ^{99m}Tc-ofloxacin to S. aureus and E.coli showed that 40 % and 30 % of radioactivity bound to bacteria respectively. It should be mentioned that in the in vitro competition assay we observed the inhibition of approximately 50% binding when 100-fold excess of unlabeled antibiotic was used as competitor.

Biological evaluation of ^{99m}Tc-ofloxacin complex was performed in mice. The results are shown in Table 1.

Clearance from the blood circulation was quite fast with <3.5 % ID/g remaining in the blood at 4 h. Moderate clearance from the kidney (16.47 ± 1.40 %ID/g at 1 h and 10.59 ± 1.21 %ID/g at 4 h post injection) followed by slow liver clearance were observed (17.57 ± 1.69 %ID/g at 1 h and 17.19 ± 1.24 %ID/g at 4 h post injection). The presence of high activity in liver and kidney suggest the hepatobiliary and urinary systems being the major route of excretion of the administered dose.

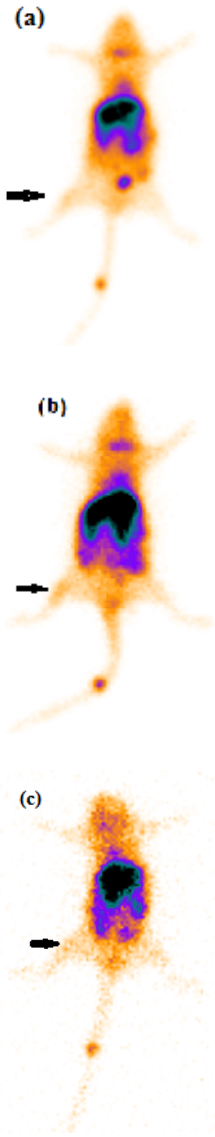

Fig 5. Scintigraphy image of mice with right thigh muscle infection (arrow show) at (a) 1 h (b) 4 h and (c) 24 h post injections.

The radioactivity concentration of infected muscle by S. aureus our E.coli at 1 h post injection was 1.96 ± 0.22 %ID/g and 1.65 ± 0.14 %ID/g which was decreased to 1.51 ± 0.11 %ID/g and 1.20 ± 0.10 %ID/g at 4 h post injection respectively. The ratio of activity in an infected muscle compared to non infected muscle was nearly two fold (T/NT = 2.33 ± 0.17 and 1.96 ± 0.13 at 1 h post injection and T/NT = 1.86 ± 0.10 and 1.48 ± 0.10 at 4 h post injection for S. aureus and E.coli respectively) which was comparable with ratio for ^{99m}Tc-ciprofloxacin (T/NT = 3.18 ± 0.10 and 1.79 ± 0.35 at 1 h and 4 h post injection in S. aureus respectively) [11]. This results show that more than 70 % of the activity was retained in infected area at 4 h post injection which may be due to the clearance of non specific uptake. At the

same time, this high retention indicates ^{99m}Tc-ofloxacin has specific affinity to both bacterial infection sites although it is higher for S. aureus infection. Scintigraphic study showed early uptake 1 h post injection for radioligand in infections sites (Figure 5). The uptake in all organs was decreased significantly after 24 h demonstrating that elimination is time depended and the early image obtained up to 4 h are probably best for detection of infection.

CONCLUSION

We have shown development and preparation of an infection imaging agent ^{99m}Tc-ofloxacin with high labeling yield. Based on the data obtained from this study, the product was stable, reproducible with high labeling efficiency with desirable characteristics making it a promising agent for imaging of infectious lesions. According to the results of in vivo biodistribution studies, we found that this complex have gram positive and gram negative affinity and a good retention time with more favorable properties than ^{99m}Tc-ciprofloxacin for detecting of infection sites, however further clinical studies are needed.

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