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

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(Received 2 January 2013, Revised 7 April 2013, Accepted 17 April 2013)

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

Published: June, 2013
http://irjnm.tums.ac.ir

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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. 99mTc-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 99mTc or 111In 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 99mTc 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 99mTc-Ciprofloxacin has been developed as a new radiopharmaceutical for preferentially diagnosing infection from sterile inflammation [14]. Solanki et al. labeled ciprofloxacin with 99mTc 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 99mTc has also been reported with this kit [16].

Ofloxacin is a broad-spectrum fluoroquinolone 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 Escherichia Coli in the current investigation, radiolabeling of the ofloxacin with 99mTc 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 (Na99mTcO4) was obtained from an in-house Mo/99mTc 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).

Radiolabelling

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 SnCl2 (20-50 µL of 2 mg/mL SnCl2, H2O in nitrogen-purged 0.1 M HCl) was added. Finally in different pH ranges 2-8, 99mTcO4 (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 99mTc-Ofloxacin was analyzed by TLC and HPLC. For the characterization of 99mTc-ligand, TLC plates were developed in ethanol: water: ammonium hydroxide (2: 5: 1 v/v/v) as well as in acetone. In the first solvent, free 99mTcO4 and 99mTc-ligand move with solvent front with Rf = 0.8-1 and the reduced technetium remain at the point of application. In the second solvent, 99mTcO4 move with solvent front with Rf = 1 and the other species remain at the point of application. The radioactivity was quantified by cutting the strip (1.5 × 10 cm2) into 1 cm pieces and counting in a well type gamma counter.

For radiochemical analysis of 99mTc-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, \( \lambda = 280 \) nm.

**Stability**

To one mL of freshly prepared human serum, we added 200 \( \mu \)L (74 MBq) of radiotracer and mixture was incubated in 37 \( ^{\circ} \)C environment. At different time points, 50 \( \mu \)L aliquots were removed and treated with 500 \( \mu \)L 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 \( \mu \)L (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 \( \mu \)L aliquots of both organic and inorganic layers were determined by use of a NaI well-type \( \gamma \)-counter. The reported octanol/water partition coefficient represents the mean (+ standard deviation) of the three measurements.

**Binding of \(^{99m}\)Tc-Ofloxacin to bacteria**

100 \( \mu \)L (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 \( 1 \times 10^8 \) colony forming units (CFU) per mL viable S. aureus or E.coli was added. The mixture was incubated for 1 h at 4 \( ^{\circ} \)C and thereafter the vials were centrifuged in a pre-cooled centrifuge for 5 min at 2000 g at 4 \( ^{\circ} \)C. 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 \( 1 \times 10^7 \) CFU bacteria into right tight muscle. After 24 h, they were injected under ether anesthesia with 100 \( \mu \)L (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.

![Chemical structure of the Ofloxacin (a). Proposed structure of\(^{99m}\)Tc-Ofloxacin complex (b).](image-url)

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 \( \mu \)g 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 SnCl₂ and different pH range in yield of labeling are showed in Figures 2 and 3, respectively.

In the radiochemical purity determination of ⁹⁹mTc-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 R₂ = 1 which belonged to ⁹⁹mTcO₄. ⁹⁹mTc-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).

The radiochemical purity of the ⁹⁹mTc-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 ⁹⁹mTc-ofloxacin is achieved.
hepatobiliary and urinary systems being the major activity in liver and kidney suggest the moderate clearance from the kidney. 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.

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

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

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.

Acknowledgements

The authors wish to thank Mr. Mirfallah and Mr. Talebi of the radioisotope department (AEIO) for providing sodium pertechnetate and assistance in quality control tests.

REFERENCES