68Ga-labeled ubiquicidin for monitoring of mouse infected with *Staphylococcus aureus*

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**Abstract.** Ubiquicidin is a cationic, antimicrobial peptide found in the innate host defence against pathogens. The peptide selectively interacts with the surface of microorganism. This work aimed to develop the radiopharmaceutical for infection imaging using antimicrobial peptide. Optimum labelling condition to obtain radiopharmaceutical purity (RCP) of 68Ga-DOTA-ubiquicidin (29-41) more than 90% was evaluated. The RCP more than 95% was achievable with 30 micrograms peptide at solution reaction pH 4. Stability testing of the labelled peptide was also studied by resembling to in vitro condition using fresh frozen human plasma. The radiolabelled peptide retained its RCP >90% after 4 hours of incubation. Animal study was performed in infected and sterile inflamed mice groups with *Staphylococcus aureus* or sterilized S. aureus at the left thigh 24 hours prior administration of 68Ga-DOTA-UBI. Radioactivity count rate at the infected or sterilized inflamed site of the left thigh of each mouse was compared and normalized with radioactivity count accumulated in normal muscle of its right thigh. Accumulation of 68Ga-DOTA-UBI in muscle of infected mice was significantly greater than the sterile inflamed mice; 4.62 ± 3.44 and 1.53 ± 1.73, respectively, at 1 hour post injection. These results showed the efficacy of 68Ga-DOTA-UBI as a radiopharmaceutical for monitoring of bacterial infection.

1. Introduction

Infectious disease is usually diagnosed based on medical history, physical examination, imaging studies, and laboratory tests. However, some infections are difficult to be diagnosed whether infected or inflamed [1,2]. Early discrimination of the infectious disease from the inflamed area is crucial for its effective treatment and for reducing the bacterial multidrug resistance from excess consumed antibiotic doses. Nuclear medicine imaging is the noninvasive technique that could be contributed for early detection of infection, especially in the case of occult infection [1,3]. To support those objectives, clinician needs to be able to discriminate between sites of sterile inflammation and those that are infected by pathogenic microorganisms. The gold standard for infection imaging is 111In or 99mTc labeled white blood cell (WBC) [4]. However, both products have the number of weakness such as requirement for labeling of individual patient’s blood sample and reinjection; need for well-trained staff and suitable facilities; the risk for infection and cross-contamination. In addition, these WBC labelling procedure has not yet extensively used. Therefore, some other products, such as an anti-neutrophil peptide, chemotactic
peptide and platelet factor, have been developed and applied for radio-imaging, yet these radiopharmaceuticals remain have some drawbacks. An antimicrobial peptide, a cationic peptide, is expressed on innate immunity against infection by a variety of pathogens [5]. The basis of the antimicrobial activity of this peptide is its interaction of the cationic domain with the negatively charged surface of the microorganism. The antimicrobial peptide of fragment ubiquicidin, UBI (29-41) (Figure 1), was labelled with $^{99m}$Tc, which targeted bacterial cells but not a sterile inflammatory process in experimental animals [6-7]. In addition, $^{99m}$Tc-UBI showed accumulation with high accuracy in fungal infections. In another study [8], the results confirmed that the accumulation of $^{99m}$Tc-UBI directly related to the viable number of bacteria as tracer accumulation in infective foci declined after administration of antibiotic medicine. Phase I clinical trial with this radiolabeled peptide showed overall sensitivity, specificity and accuracy of 100%, 80% and 94.4%, respectively [9,10].

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\text{Figure 1. Chemical structure of UBI fragment (29-41).}
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As better sensitivity and specificity of positron emission tomography (PET) compared to single photon emission tomography (SPECT), infection imaging with PET become popular increasing. [11–13]. Ebenhan et al., [14] conjugated UBI fragments to a trivalent metal chelator, 2-[4, 7-bis(carboxymethyl)-1, 4, 7-triazonan-1-yl] acetic acid (NOTA), and labeled with $^{68}$Ga PET radionuclide. The $^{68}$Ga-NOTA-UBI was investigated in a rabbit infection model. They found that the tracer could differentiate the infected muscle from the sterile inflamed site with the ratio of infected versus inflamed 3.5 ± 0.86 at 1-hour post injection. Viche et al., [15] was also evaluate the efficacy of $^{68}$Ga-NOTA-UBI for PET imaging in mice infected with \textit{S. aureus} and confirmed the selectivity of this tracer to the infected area compared to the inflamed site with a ratio of target to non-target (T/NT) 5.0 at 1 hour. Based on the previous studies of preclinical and clinical data, exhibit the efficacy of UBI as a radiopharmaceutical to differentiate bacterial infection from sterile inflammation. This research aims to develop a radiopharmaceutical of UBI and assess its potential for PET infection imaging. UBI was conjugated to DOTA chelating ligand and labeled with a trivalent metal PET radioisotope, Gallium-68 ($^{68}$Ga).

2. Material and method
Ubiquicidin (29-41) conjugated with 2-[4, 7, 10-tris(carboxymethyl)-1, 4, 7, 10-tetraacycloclodec-1-yl] acetic acid (DOTA) was purchased from ABX, Germany. Radionuclide of $^{68}$Ga was eluted from $^{68}$Ge/$^{68}$Ga generator, which obtained from Eckert & Ziegler, Cyclotron Russia. Fresh frozen human plasma was available from the Pathology Department, Faculty of Medicine Srinakharinwirot University. Other chemicals were Sigma-Aldrich products and used as received.
2.1. Radiolabeling of DOTA UBI with $^{68}$Ga
A 10-mL vial containing 30 µg DOTA-UBI was added with aliquots of 200 µL 1M NaOAc pH 5.5, and 20 µL ascorbic acid (100 mg/mL). A 1.0-2.0 mL of $^{68}$GaCl$_3$, collected from the $^{68}$Ge/$^{68}$Ga generator, was added into the reaction vial. The pH of reaction solution was checked to confirm ranged between 4.0-4.5. The reaction vial was heated at 100 °C for 15 minutes and then waited for cooling down to room temperature for 5 minutes. Radiochemical purity (RCP) of $^{68}$Ga-DOTA-UBI was determined by HPLC equipped with a radioactivity detector (Agilent 1200 with GABI software).

2.2. In-vitro stability testing
The 50 µL of $^{68}$Ga-DOTA-UBI was pipetted into four of 1.5-mL microcentrifuge tubes. Each tube was added with 50 µL of fresh frozen human plasma and then incubated at 37 °C for 1, 2, 3 and 4 hours. The tube was then added with 50 µL of 96% ethanol, 25 µL of 1 mg/mL 2-[Bis[bis(carboxymethyl)amino]ethyl]amino]ethyl]amino]acetic acid (DTPA) in 0.4 M NaOAc at each time point. The supernatant of $^{68}$Ga-DOTA-UBI was separated from precipitation by centrifugation at 5000 rpm for 5 min before analysis of RCP by HPLC.

2.3. Protein binding
Binding of $^{68}$Ga-DOTA-UBI to proteins was determined by molecular exclusion using permeable membrane columns (Illustra micro spin G-50, GE Healthcare). An aliquot of $^{68}$Ga-DOTA-UBI was taken out and diluted to 1:10 with 10% human serum albumin (HSA) in a 1.5 mL microcentrifuge tube. The tubes were incubated at 37 °C for 30, 60, 90 and 120 minutes. Another tube containing 0.05 M PBS pH 7.4 was done in parallel as a control experiment. At the finished each time point, each tube was centrifuged with micro spin G-50 at 5000 rpm for 5 minutes to separate protein-bound fraction from unbound fraction. Radioactivity count rate of the two parts was measured with a scalar rate meter (Ludlum model 2200).

2.4. Biodistribution of $^{68}$Ga-DOTA-UBI in infected and inflamed mice
Animal experiment was approved by the Ethical Committee for the Use and Care of Animals in Scientific Research of Thailand Institute of Nuclear Technology and was performed according to the Animals for Scientific Purposes Act, B.E. 2558 (A.D. 2015). Two groups (infection and inflammation), every 3 animals, specific pathogen free (SPF), of JcL:ICR male mice, aged 8 weeks, were used. Staphylococcus aureus (S. aureus) TISTR 2329, obtained from the Thailand Institute of Scientific and Technological Research (TISTR), was used as the infecting agent.

The animals were divided into 2 groups. First group, infection was induced by inoculation with 0.1 mL of S. aureus (1x10$^9$ cfu/mL) at the left upper thigh muscle. Second group, sterile inflammation was induced by inoculated with 0.1 mL of moist treated S. aureus (1x10$^9$ cfu/mL) at the same region of the left thigh. All inoculated mice were housed under pathogen-free 24 hours prior administration of $^{68}$Ga-DOTA-UBI. Each mouse was injected with 0.1 mL of $^{68}$Ga-DOTA-UBI (37 MBq/mL in 0.9% NaCl) via the tail vein. After 1 hour of administration, mice were sacrificed by inhalation with isoflurane. All tissues, samples of blood and thigh muscle (left as the target, right as control) of each mouse were collected, weighed and counted for radioactivity (Ludlum model 2200 with well type detector). The percentage of injected dose per gram of tissue (% id/g) of each mouse was calculated. Ratio of target to non-target (T/NT) of infected or inflamed at the left thigh versus the right thigh muscle of each mouse was determined to compare the specificity of the radiopharmaceutical.
3. Results and discussions

3.1. Radiolabelling of $^{68}$Ga with DOTA UBI

For optimum pH, recommended range of pH between 3.5 and 4.5 is used as in the bibliography [14-15]. Effect of ascorbic is also tested as a reducing agent and a trans-chelating to achieve robust condition and to prevent the colloidal formation of $^{68}$Ga. The Optimum condition for labelling $^{68}$Ga with DOTA-UBI was 30 µg of UBI peptide, at reaction pH 4.0-4.2 with adding of 2 mg ascorbic acid. Normally, radiochemical purity of $^{68}$Ga-DOTA-UBI was >95% with minimal degradation peak of UBI peptide at retention time of 5 min as in Figure 2.

![Figure 2](image)

**Figure 2.** HPLC radio-chromatogram of $^{68}$Ga-DOTA-UBI labelled solution showed retention time at 5.75 minutes (97.69% ± 1.05, n=15). Luna C18, 5 µm 100 Å, 150x4.6 mm, gradient system; 0-2 min 10% B, 2 – 12 min 10% B – 70% B, 12 – 13 min 70% B, 13 – 15 min 70% B – 10% B; A=0.1% TFA/H2O, B= 0.1% TFA/CH3CN.

3.2. In vitro stability testing

The radiochemical purity of $^{68}$Ga-DOTA-UBI was slowly declined however, it remained >90% at 4 hours of incubation. This result confirmed that the stability of $^{68}$Ga-DOTA-UBI was good enough to use as a radiopharmaceutical for internal imaging, (Figure 3).

![Figure 3](image)

**Figure 3.** Stability testing of $^{68}$Ga-DOTA-UBI in fresh frozen human plasma showed %RCP > 90% up to 4 hours of incubation.

3.3. Protein binding

Binding of $^{68}$Ga-DOTA-UBI with protein ranged from 50-65% as shown in Figure 4.
3.4. Preliminary Biodistribution

The optimum biodistribution study was performed 1 hour post administration as the previously study by Vilche [15]. Accumulation and excretion data of $^{68}$Ga-DOTA-UBI in infected mice was also compared to the sterile inflamed mice and showed intense radioactivity in the kidney (>30% id/g) and negligible uptake in the liver. However, most of the radioactivity was excreted via urinary bladder (>98%). Biodistribution data of $^{68}$Ga-DOTA-UBI expressed as a percentage of radioactivity per gram of each organ, (%id/g excluded of kidney and bladder), as shown in Figure 5. The result was in agreement with those data of $^{68}$Ga-NOTA-UBI studied by Ebenhan [14] and Vilche [15]. Different of T/NT ratio in the infected and the inflamed area was observed. T/NT ratio of the infected mice was higher than the inflamed mice, 4.62±3.44 versus 1.53±1.73, respectively. The number of S. aureus determined by the Thai irradiation centre, TINT (AOAC 2003.11:2015) found 1.6x10$^7$-3.6x10$^7$ and <10 colonies per gram of the infected and the inflamed muscle, respectively. This result confirmed that the DOTA, when conjugation to the UBI peptide, did not change labelling and antimicrobial activity of the peptide. The radiolabelled peptide preserved its bioactivity which expressed by selectively interact with the bacterial at the infected site compared to inflammation tissue.

Figure 4. Protein binding of $^{68}$Ga-DOTA-UBI at 30, 60, 90 and 120 minutes.

Figure 5. Biodistribution of $^{68}$Ga-DOTA-UBI of infected compared to inflamed mice 1 hour post administration.
4. Conclusion

Labelling of DOTA-UBI with $^{68}$Ga radionuclide under the optimized condition has given RCP $>95\%$ without further purification process. The labelled peptide was intact under in vitro condition up to 4 hours. Accumulation of $^{68}$Ga-DOTA-UBI was rapid with rather high radioactivity background in circular blood system, however mainly of radioactivity was later excreted via kidney. The radiolabelled peptide could be discriminate pathogen infection from sterile inflammation which means the potential for clinical use. Imaging with PET radiopharmaceutical of UBI peptide is an invasive technique that is suitable for the detection of deep muscle infection or osteomyelitis infection. Further clinical evaluation of this radiopharmaceutical should be performed to ensure its potential clinical diagnostic imaging.

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