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Three different procedures in labeling of Ubiquicidin with technetium 99m: a comparative study

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Background: UBI 29-41 (a derivative of antimicrobial peptide ubiquicidin) labelled with ^{99m}Tc is reported to discriminate between bacterial infections and sterile inflammatory processes. In this study, three lyophilized kit were performed, one of them based on the direct labelling with only $SnCl_2$ as reducing agent, and other two based on 6-hydrazinopyridine-3-carboxylic acid (HYNIC) and tricine as a coligands with or without ethylenediamine -N,N'-diacetic acid (EDDA). ***Materials and Methods:*** Synthesis of UBI 29-41 was performed on solid phase using a standard Fmoc strategy. BOC-HYNIC was conjugated with peptide in solution. Three lyophilized kits were prepared as follows: kit 1: 40 µg UBI 29-41, 5 µg $SnCl_2$, pH = 9; kit 2: 40 µg UBI 29-41, 40 µg $SnCl_2$, 20 mg tricine, pH = 5.2; kit 3: 40 µg UBI 29-41, 40 µg $SnCl_2$, 15 mg tricine, 5 mg EDDA, pH = 7. With addition of $^{99m}TcO_4^-$ solution, kits were labeled under specific conditions, and the radiochemical purity was evaluated by ITLC and HPLC methods. Stability and protein binding in human serum followed by *in vitro* binding to bacteria were assessed. Biodistribution of radiopeptides in *staphylococcus aureus* infected rats muscles were studied using *ex vivo* counting and scintigraphy. ***Results:*** Radiochemical analysis indicated rapid and high labeling yield (>95%) for the three kits. Binding to bacteria for kit 2 was to some extent higher than that was obtained for the two other kits. Specific accumulation in infected thigh muscles, as indicated by T/NT ratios was 3.29, 4.6 and 3.77 for kit 1, 2 and 3, respectively. ***Conclusion:*** The HYNIC-UBI 29-41 labeled in presence of tricine as coligands (kit 2) showed the most promising results for further *in vivo* evaluation. **Iran. J. Radiat. Res., 2009; 7 (2): 97-104**

Keywords: *Infection, antimicrobial peptide, ^{99m}Tc, direct labelling, indirect labelling.*

INTRODUCTION

In several previous studies, the ^{99m}Tc-labelled cationic antimicrobial peptide derived from human ubiquicidine (UBI) was introduced for the detection of bacterial and fungal infections in animals [1-6]. Originally, UBI was isolated from murine macrophages [7, 8] and later from human airway epithelial cells. UBI 29-41 is a cationic human antimicrobial peptide fragment (MW 1.69 kDa) with the amino acid sequence Thr-Gly-Arg-Ala-Lys-Arg-Arg-Met-Gln-Tyr-Asn-Arg-Arg, and, therefore, with 6 positively charged residues and no cysteines. Many approaches have been used to radiolabel peptides with ^{99m}Tc. Usually these methods follow one of two strategies: direct or indirect labeling. It is generally thought that by direct labeling, the ^{99m}Tc is bound to sulfydryls produced by the reduction of the cysteine bridge in a peptide. Very little is known about the number of donor atoms and the coordination geometry around the ^{99m}Tc center. The indirect labeling method involves the use of a bifunctional chelating agent (BFCA) to incorporate the ^{99m}Tc into the peptide [9-11]. The advantages of this labeling method include a well defined chemistry and the possibility of post conjugation labeling in which the peptide is first conjugated with a BFCA and stored until required for radiolabeling. ^{99m}Tc-labelled UBI-derived peptides can be good candidates for radiolabelling and studies on detection of bacterial infections in humans [12, 13].

Application in humans requires standardization of the radiolabelling. Diverse dry kits of this peptide were developed in the present work for ^{99m}Tc labelling. The aim of the present study was to evaluate and compare the radiochemical and biologi-

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cal characteristics of three different kits in ^{99m}Tc labeling of UBI 29-41.

MATERIALS AND METHODS

All chemicals were obtained from commercial sources and used without further purification. Tritylchloride resin was obtained from NovaBiochem. The prochelator HYNIC-Boc was synthesized according to Abrams *et al.* [14]. The reactive side chains of the aminoacids were masked with one of the following groups: Arg, 2, 2, 4, 6 ,7 - pentamethyl-dihydrobenzofurane-5-sulfonyl; Asn, triphenylmethyl; Gln, triphenylmethyl; Lys, t-butoxycarbonyl; Tyr, t-butyl; Thr, t -butyl. For sterility filtration, 20-µm Millex-GS filters from Millipore were used. Sodium pertechnetate (Na $^{99m}TcO_4$) obtained from commercial ^{99}Mo/ ^{99m}Tc generator (Radioisotope Division, AEOI).

Analytical reverse phase-high performance liquid chromatography (RP-HPLC) was performed on a JASCO 880-PU intelligent pump HPLC system equipped with a multi-wavelength detector and a flow-through Raytest-Gabi γ-detector. CC250/4 Nucleosil 120-3C18 column from Macherey-Nagel were used for HPLC. The gradient systems consisted of 0.1% trifluoroacetic acid (TFA)/ water (solvent A), acetonitrile (solvent B), flow: 1 mL/min, λ = 280 nm. Quantitative gamma counting was performed on ORTEC Model 4001 M γ-system well counter.

Synthesis

The peptide was synthesized by standard Fmoc solid-phase synthesis on tritylchloride resin with substitution of 0.8 mmol /g. Coupling of each amino acid was performed in the presence of 3 molar excess of Fmoc-amino acid, 3 molar excess of *N*-hydroxybenzotriazole (HOBT), 3 molar excess of diisopropylcarbodiimide (DIC), and 5 molar excess of *N*-etthyldiisopropylamine (DIPEA) in *N*-methylpyrrolidone (NMP) for 2 h. Completeness of coupling reactions was monitored by the Kaiser test and the Fmoc groups were removed by adding 20% piperidine/*N,N*-dimethylformamide (DMF). For HYNIC conjugation, the fully protected peptide was cleaved from the resin with 20% acetic acid. 1.2 mol HYNIC-Boc was coupled with 1.2 molar of O-(7-azabenzotriazol-1-yl)-1,1,3,3,tetramethyluronium hexafluorophosphate (HATU) to the N-terminus of peptide. After deprotection and precipitation, the products were purified and characterized by RP-HPLC and ESI-MS.

Preparation of kits

The compositions of the 3 types of lyophilized kits per vial were as follows - kit A: 40 µg UBI, 5 µg $SnCl_2$ (2.5 µL of 2 mg/mL $SnCl_2$, $2H_2O$ in nitrogen purged 0.1 M HCl), in distilled water final pH 9; kit B: 40 µg HYNIC-UBI, 40 µg $SnCl_2$ (10 µL of 2 mg/mL $SnCl_2$, $2H_2O$ in nitrogen purged 0.1 M HCl), 20 mg tricine in sodium acetate buffer 0.5 M with the final pH 5.2; kit C: 40 µg HYNIC-UBI, 40 µg $SnCl_2$ (10 µL of 2 mg/mL $SnCl_2$, $2H_2O$ in nitrogen purged 0.1 M HCl), 15 mg tricine, 5 mg ethylenediamine-N,N'-diacetic acid (EDDA) in distilled water with final pH 7. Parameters of lyophilization: -40°C: 21 h, -40°C to + 25°C: 13 h, + 25°C: 14 h, total time: 48 h, vacuum: 0.12 mbar, final temperature: 20 °C. Kits were sealed in N_2 atmosphere.

Labeling procedure

Kit A was reconstituted and labelled by adding 555 MBq $^{99m}TcO_4^-$ in 0.2 mL saline followed by gently stirring at room temperature for at least 1 h. Radiolabeling of kits B and C were performed by adding 0.5 mL 0.9% saline in an evacuated vial and the mixtures were allowed to preincubate for 5 min. Then, 555 MBq $^{99m}TcO_4^-$ in 0.5 mL saline was added to the vials and incubated for 10 min at 100°C and then cooling down to room temperature for 10 min.

Quality control

The reaction mixtures after labeling of UBI or HYNIC-UBI were analyzed by ITLC and reverse-phase HPLC. ITLC was performed on silica gel 60 (Merck) using

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different mobile phases. 2-Butanone for free $^{99m}TcO_4^-$ (R_f = 1), 0.1 M sodium citrate pH 5 to determine the nonpeptide-bound ^{99m}Tc coligand and $^{99m}TcO_4^-$ (R_f = 1) and methanol/1 M ammonium acetate 1/1 for ^{99m}Tc- colloid (R_f = 0).

In HPLC analysis a gradient with two different eluents, 0.1% trifluoroacetic acid (TFA)/water (A) and acetonitrile (B), at a flow rate of 1mL/min and λ = 280 nm was used. The gradient was performed as follows: 0 min 95% A (5% B), 5 min 95% A (5% B), 20 min 0% A (100% B), 23 min 0% A (100% B), 25 min 95% A (5% B).

Stability and protein binding

Stability and serum protein binding of ^{99m}Tc-labelled UBI 29-41 and HYNIC-UBI 29-41 were challenged by adding 1 mL of radiolabeled peptide with activity between 370-3700 MBq to a vial contain 1ml fresh human serum. The reaction mixtures were incubated at 37°C for 24 h and analyzed by ITLC and PD10 column (Pharmacia, Upsala, Sweden). After washing the column with PBS containing 0.1% BSA, the activities bound to serum protein and peptide was measured with a well-type gamma counter.

In vitro binding

An aliquot of *S. aureus,* at a concentration of 1 × 10^8 colony forming units (CFU)/mL, was prepared and stored at -20°C to be thawed immediately before use. Binding of ^{99m}Tc-labelled UBI 29-41 and HYNIC-UBI 29-41 to bacteria was assessed at 4°C. A mixture containing 1/10 of ^{99m}Tc-labelled peptides in 15 mM sodium phosphate buffer pH 7.5 was transferred to an Eppendorf vial. 0.1 mL of Na-PB containing approximately 2 × 10^7 viable bacteria was added. The mixture with a final pH of 5 was incubated for 1 h at 4°C and thereafter the vials were centrifuged in a pre-cooled centrifuge at 2,000 × *g* for 5 min. The supernatant was removed and the radioactivity in the bacterial pellet was determined in a dose calibrator.

Animal studies

Animal experiments were performed in compliance with the regulations of NSTRI, and with generally accepted guidelines governing such work. Male Swiss mice, weighing 25-30 g were infected by injecting 0.1 mL of saline containing 2 × 10^7 CFU bacteria into right thigh muscle. After 24 h rats were injected with 20 MBq of 0.35 nmol (0.5 µg total peptide mass) of radiolabeled peptide in saline into the tail vein. For *ex vivo* counting mice were sacrificed after 1, 2 and 4 h and infected and non-infected thigh muscles followed by various organs were dissected, weighed and counted for radioactivity. Data were expressed as the percentage of injected dose per gram of tissue (%ID/g).

RESULTS

The peptide was synthesized in an overall yield of 50%. Coupling of HYNIC to the N- terminus of peptide was performed with yield of 30% (figure 1). The purity was >95%, as confirmed by the HPLC method. Radiochemical analyses showed high labeling yield (95-98%) for all kits, as assessed by ITLC and HPLC at a specific activity of 50 GBq/µmol that were stable for 24 hours (figure 2). The HPLC elution times for three formulations were as follow: kit A: 5.9 min for $^{99m}TcO_4^-$ and 20.2 min for ^{99m}Tc-UBI, kit B: 5.6 min for ^{99m}Tc-tricine and 18.2 min for ^{99m}Tc-tricine-HYNIC-UBI and kit C 5.6 min for ^{99m}Tc-EDDA or ^{99m}Tc-tricine and 19.7 min for ^{99m}Tc-EDDA-tricine-HYNIC-UBI. None of the preparation methods required further purification steps to give high radiochemical purity products which retained more than 90% of their initial activity 24 h after preparation.

The different preparation process of ^{99m}Tc-UBI 29-41 revealed small amounts of released free ^{99m}Tc after incubation in

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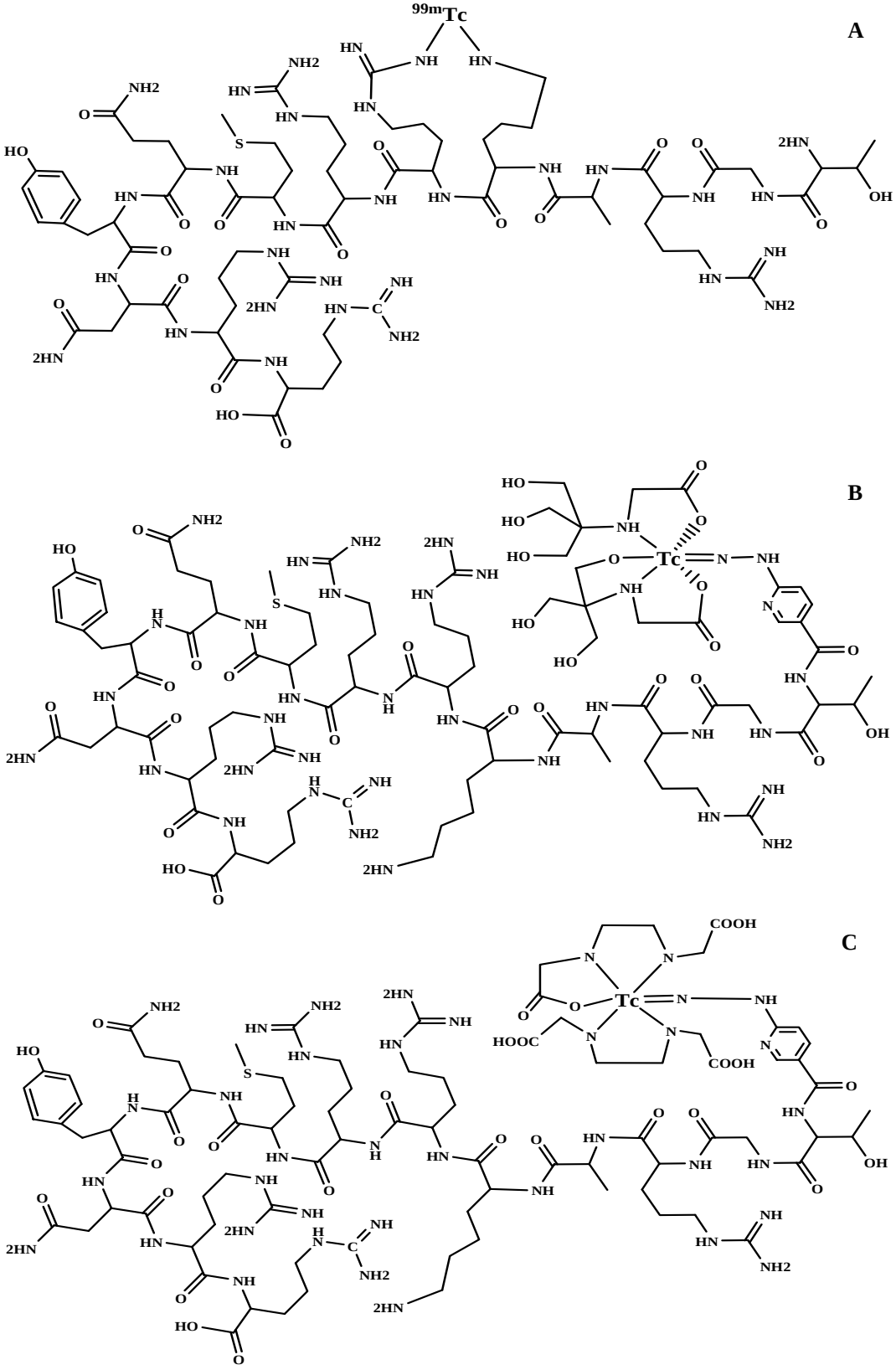

Figure 1. Structural formula of (a) ^{99m}Tc- Ubiquicidin 29-41 (kit A); (b) ^{99m}Tc/Tricine/HYNIC-Ubiquicidin 29-41 (kit B); (c) ^{99m}Tc/Tricine/EDDA/HYNIC-Ubiquicidin 29-41 (kit C)

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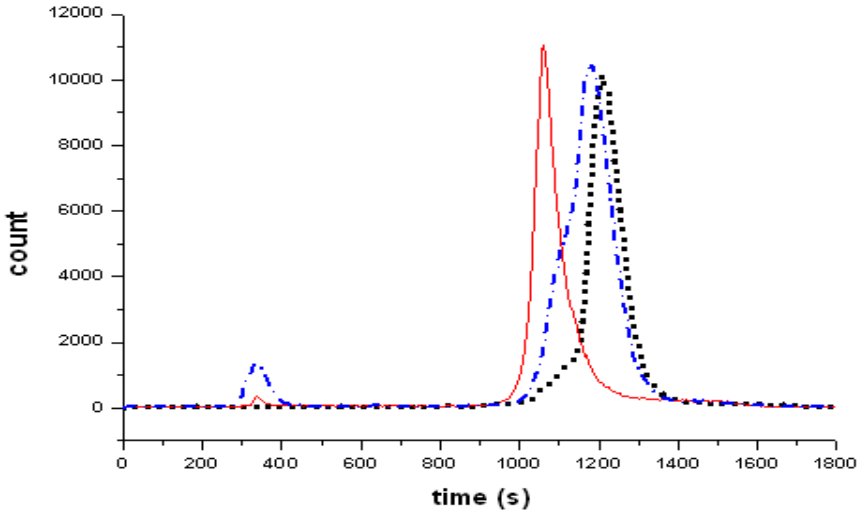

Figure 2. RP-HPLC analysis after labeling with ^{99m}Tc for kit A (dot); kit B (solid) and kit C (dash dot).

human serum. The amount of ^{99m}Tc that was displaced from the labelled peptides to human serum after 24 hour was 20% (kit A), 30% (kit B) and 10% in kit C. Results for *in vitro* testing showed binding of 75% (kit A), 85% (kit B) and 70% (kit C) of the added activity to bacteria. The obtained data shows that although using tricine alone as a coligand have a slightly higher protein binding in serum in compare with direct labeling and tricine/EDDA as a coligands, binding affinity to bacteria for tracer when labeled with tricine is some extent higher than that was obtained for two another method. This showed that in kit B, there were lower interactions with biologically active sites of the peptide, as it might hamper its biological activities.

After injection into infected mice, all tracers were rapidly removed from circulation by renal excretion (5-22%ID/g) that at 1 h amount for kit B was higher than two another's kit (figure 3). Although small amount of activity were observed in the liver, intestine, spleen and thyroids, in 1 h post injection activity in liver and intestine, for kit A was more than kits, B and C. Specific accumulation in infected

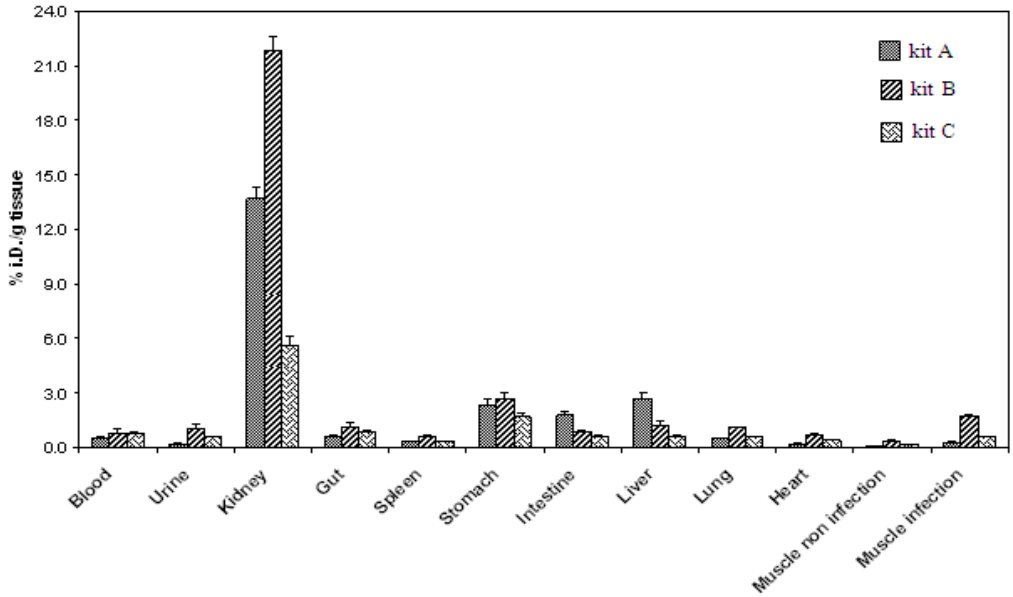

Figure 3. Biodistribution in mice with thigh muscle infection for kit A, kit B and kit C 1 h after injection. Data are presented as %ID/g±SD and results are the means of groups of four animals.

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thigh muscles, as indicated by T/NT ratios, were 3.29, 4.6, 3.77 at 1 h after injection for kit A, B and C, respectively. Due to fast renal excretion after 2 and 4 h, post injection activity in organs was reduced considerably.

Based on higher T/NT ratios for tracer labeled with method B Scintigraphy in mice's with thigh muscle infection performed only for kit B at 60 min post injection (figure 4). A high uptake of activity in infection site compared with normal site was observed.

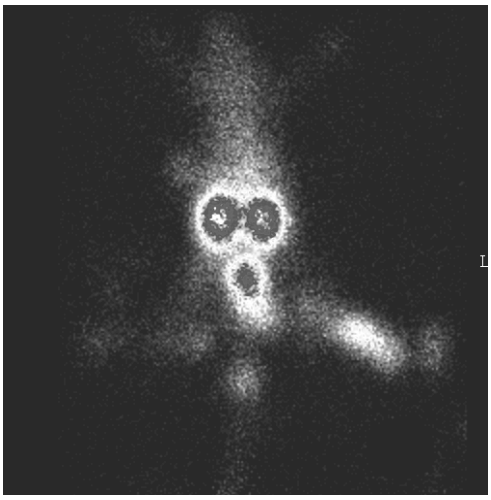

Figure 4. Typical Scintigram of mice with thigh muscle infection at 60 min after injection of ^{99m}Tc/Tricine/HYNIC-Ubiquicidin 29-41 (kit B).

DISCUSSION

It should be realized that Ubiquicidin 29-41 can be labeled with ^{99m}Tc using a different labeling techniques consisted of direct and indirect methods [6,9,10]. In the present study we prepared three freeze-dried kits based on two methods. Alteration in amount of peptide per each kit (40 µg), followed by modification in constituent and labeling procedure were performed in comparison with pervious studies [6, 9, 10].

The results showed that UBI 29-41 and HYNIC-UBI 29-41 could be labeled by a simple kit procedure with high specific activity. The present results revealed that labeled peptide in all three formulation bind to bacteria followed by observation of inhibition of binding in present of excess cold peptide as a competitor. These results were comparable with those obtained by Nibbering *et al.* [3] and Welling *et al.* [9, 10]. They used the same radiopharmaceutical to study its biodistribution, and observed binding of about 50% and T/NT ratio of 1.84 ± 0.27 (% ID/g) after 2 h.

In earlier studies UBI 29-41 labeled via direct method had been used with two different concentrations of 400 µg and 50 µg of peptide [12, 13]. In this study the amount of peptide was reduced to 40 µg per freeze-dried kits to obtain a solution with higher ratio of labeled peptide to unlabeled peptide. Since the results of the study showed that the binding of radiolabeled peptide was reduced by a pre-incubation with unlabeled peptide, by reducing the amount of peptide a solution with higher ratio of labeled peptide to unlabeled peptide was achieved, and it was assumed by employing such solution higher T/NT ratios could be obtained.

Previous studies have indicated that HYNIC acts as a Monodentate or bidentate ligand to form a mixed ligand ^{99m}Tc complex in the presence of appropriate coligands [15, 16, 17]. The use of coligands allows easy modification of the hydrophilicity and pharmacokinetics of ^{99m}Tc-labeled peptide conjugates.

Tricine continues to be one of the most versatile coligands for ^{99m}Tc-HYNIC peptide conjugates. The use of tricine as a coligand to produce ^{99m}Tc-HYNIC conjugates has been studied extensively. Liu and Edwards [18] have reported that the use of tricine as coligands suffers from the instability of technetium complexes in the absence of excess coligand [19]. Furthermore, they have reported that the use of lower tricine concentrations (<10 mg/mL) results in the formation of a significant amount of ^{99m}Tc-colloid [19]. In this study it has been attempted to avert those problems previously reported in the literature by using a greater concentration of tricine (20 mg/mL) at a pH of 5-6. When tricine was

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used as a stand-alone coligand (kit B), the formation of radiocolloid (2%) was only minimally present as indicated by ITLC and is an improvement over other literature Preparations.

Early studies of Decristoforo and co-workers [20], employed either tricine or EDDA as coligands in the radiolabeling of Tyr^3-octreotide (TOC) with ^{99m}Tc. Decristoforo has recently reported this exchange type of labeling to be very effective in producing ^{99m}Tc-conjugates of [D-Glu^1]-Minigastrin [21]. In our study Data show that also kit C (tricine/EDDA) has lower human serum protein binding, but binding to bacteria and T/NT ratio in mice were reduced using EDDA in formulation (kit C). Also in kit A (direct method) binding to bacteria was lower than binding for kit B (indirect/ tricine) that could be done to replace of ^{99m}Tc at or nearby a biologically active site of peptide.

In biodistribution study for indirect method (kit B), the percentage of administered dose for kidneys was to some extent higher and for liver it was slightly lower than reported by direct method (kit A) [2, 4 ,10], possibly because the achieved complex was more hydrophilic than the complex which was prepared by direct method.

Although the biodistribution results show fast renal excretion for all methods, moderate accumulation of tracer labeled with method A in the liver and intestine indicates which was less attractive for imaging of abdominal infections. Scintigrphy was in agreement with our pervious findings with respect to the favorable biodistribution profile of the labeled peptide and its rapid accumulation at sites of bacterial infection that in mice's with thigh muscle infection [1, 5]. The rapid visualization of infections indicating that this peptide directly tag the bacteria at the site of infection. Based on the radiochemical and biological pattern and since UBIwas a human peptide, it is believed that the prepared lyophilized kit B could have been the candidate of choice for studies on detection of bacterial infections in humans.

CONCLUSION

Both peptides UBI and HYNIC-UBI can be prepared in the dry kit form for labeling with ^{99m}Tc. On the basis of these results, the HYNIC-UBI 29-41 kit without EDDA (kit B) seem to be the most promising candidates for infection imaging.

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