

New Peptide Based Freeze-Dried Kit [^{99m}Tc -HYNIC]-UBI 29-41 as a Human Specific Infection Imaging Agent

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ABSTRACT

Introduction: Ubiquicidin 29-41 (UBI) is a fragment of the cationic antimicrobial peptide that is present in various species including humans. The purpose of this study was to investigate radiochemical and biological characteristics of [6-hydrazinopyridine-3-carboxylic acid (HYNIC)]-UBI 29-41 designed for the labeling with ^{99m}Tc using tricine as coligand.

Methods: Synthesis was performed on a solid phase using a standard Fmoc strategy and HYNIC precursor coupled at the N-terminus. Purified peptide conjugate was labeled with ^{99m}Tc at 100°C for 10 min. Radiochemical analysis involved ITLC and high-performance liquid chromatography methods. Peptide conjugate stability and affinity to human serum was challenged for 24 hours and its *in vitro* binding to bacteria was assessed. Biodistribution and accumulation of radiopeptide in *staphylococcus aureus* infected mice were studied using scintigraphy and *ex vivo* counting.

Results: Radiolabeling was performed at high specific activities, and radiochemical purity was >95%. The stability of radiolabeled peptide in human serum was excellent. *In vitro* studies showed 70% of radioactivity was bound to bacteria. After injection into mice with a bacterial infection, removing from the circulation occurred mainly by renal clearance and site of infection was rapidly detected within 30 min. Target to nontarget muscle ratio was $2.099 \pm 0.05\%$ at 30 min post injection.

Conclusion: [^{99m}Tc -HYNIC]-UBI 29-41 showed favorable radiochemical and biological characteristics which permitted detection of the infection with optimal visualization within 30 min.

Key Words: Antimicrobial peptide; HYNIC; Kit; Ubiquicidin

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INTRODUCTION

In the last decade, strong efforts have been undertaken to establish radiopeptides in nuclear medicine for receptor-specific diagnosis or therapy (1, 2). Recently, antimicrobial peptides labeled with direct or indirect methods have been proposed as new ^{99m}Tc agents to distinguish bacterial infections from sterile inflammatory processes (3-10). 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 sulfhydryls 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. 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. We considered labeling of this peptide through the bifunctional chelators and among them HYNIC is more attractive because of its monodenticity that may allow the use of a variety of coligands, leading to quite different biodistributions (11, 12).

In this study, we have used a new ^{99m}Tc-labeled HYNIC-UBI 29-41 freeze-dried kit formulation for evaluation of biological activity profile and detection of infection sites in mice injected with live *S. aureus*. As a control, inflammation was induced by sterilized turpentine oil. As a difference from previous studies (9, 10), our freeze-dried kit contains a greater quantity of HYNIC-UBI 29-41 (40 µg) with some modifications in another constituent (40 µg SnCl₂, without adding mannitol and using acetate buffer 0.5 M, pH 4 as a solvent) and also difference in labeling procedure (100°C for 10 min).

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 (13). 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}TcO₄) was obtained from commercial ⁹⁹Mo/ ^{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 multiwavelength 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: 0.75 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*-ethyl-diisopropylamine (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). 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, product was purified and characterized by RP-HPLC and ESI-MS.

Kit formulation

1 mL solution of sodium acetate buffer 0.5 M with the final pH 4, containing 20 mg tricine, 40 µg SnCl₂ (20 µL of 2 mg/mL SnCl₂, 2H₂O in nitrogen purged 0.1 M HCl) and 40 µg purified HYNIC-peptide conjugate, was filtrated into a glass vial and freeze dried.

Labeling and quality control

Radiolabeling of kit was performed by adding 0.5 mL 0.9% saline in an evacuated vial and the mixtures were allowed to preincubate for 5 min. Afterwards 555 MBq ^{99m}TcO₄⁻ in 0.5 mL saline was added to the vial and incubated for 10 min at 100°C. After cooling to

room temperature, the labeled peptide was analyzed by analytical HPLC and ITLC on silica gel 60 (Merck) using different mobile phases. 2-Butanone for free ^{99m}TcO₄⁻ (R_f = 1), 0.1 M sodium citrate pH 5 to determine the nonpeptide-bound ^{99m}Tc coligand with ^{99m}TcO₄⁻ (R_f = 1) and methanol/1 M ammonium acetate 1/1 for ^{99m}Tc- colloid (R_f=0).

Stability and affinity to human serum

The stability and affinity of [^{99m}Tc-HYNIC]-UBI 29-41 was challenged by adding 1 ml of radiolabeled peptide with activity between 10 and 20 mCi to a vial contain 1ml fresh human serum. The reaction mixtures were incubated at 37°C for 24 h and analyzed by ITLC for stability and placing 100 µl of reaction mixture on a PD10 column to evaluate affinity to plasma proteins. After washing the column with PBS contain 0.1% BSA activity bound to serum protein and labeled peptide was measured with well-type gamma counter.

In vitro binding assay

Over night cultures of *S. aureus* were prepared in brain-heart infusion broth (Oxoid, Basingstoke, UK) in a shaking water-bath at 37°C. The next day, bacteria were washed, counted, aseptically aliquoted at a concentration of 1 × 10⁸ colony forming units (CFU)/mL, stored at -20°C and thawed immediately before use. Binding of ^{99m}Tc-labeled peptide to bacteria was assessed at 4°C. In short, 0.1 mL of 15 mM sodium phosphate buffer (Na-PB, pH 7.5) containing 1/10 of the preparation containing labeled peptide was transferred to an Eppendorf vial. Next, 0.8 mL of 50% (v/v) of 0.01 M acetic acid in Na-PB containing 0.01% (v/v) Tween-80 and 0.1 mL of Na-PB containing approximately 2 × 10⁷ viable bacteria were 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 bacterial pellet was gently resuspended in 1 mL of Na-PB and re-centrifuged as above. The supernatant was removed and the radioactivity in the bacterial pellet was determined in a dose calibrator. The radioactivity associated with bacteria was expressed as percentage of added ^{99m}Tc activity bound to 2 × 10⁷ of viable bacteria.

Animal Studies

Male Swiss mice, weighting 25-30 g were infected by injecting 0.1 mL of saline containing 2 × 10⁷ CFU bacteria into right thigh muscle. After 18 h rats were injected under ether anesthesia with 20 MBq of 0.35 nmol [^{99m}Tc-HYNIC]-UBI 29-41, in saline into the tail vein. For comparison, a sterile inflammation was

induced by an intramuscular injection of 0.1 mL of sterile saline containing 100 µg of turpentine oil 24 h preceding administrations of the tracers. At 1 h after injection, accumulation of the tracer in infected or inflamed areas was assessed by planer scintigraphy under ether anesthesia. For ex vivo counting's, mice's were sacrificed after 30 min, 1 and 2 h, and organs of interest were collected, weighed and radioactivity was measured in a γ-counter.

RESULTS

Synthesis and radiolabeling

After standard solid-phase synthesis of the peptide, coupling of HYNIC-Boc to the N-terminus of peptide was performed in the solution in an overall yield of 40% (Fig. 1).

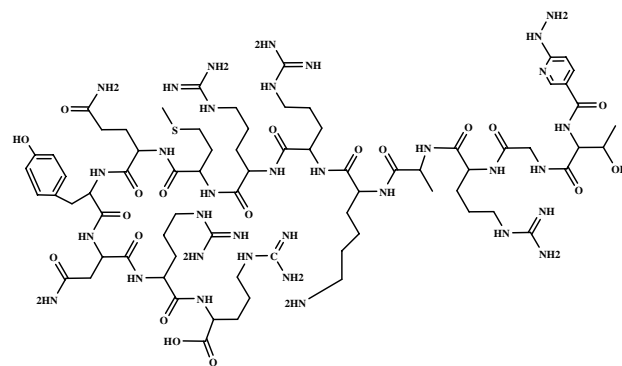


Fig. 1: Structural formulae of [HYNIC]-UBI 29-41

The composition and structural identity of [HYNIC]-UBI 29-41 were verified by analytic HPLC and ESI-MS (Fig. 2, 3).

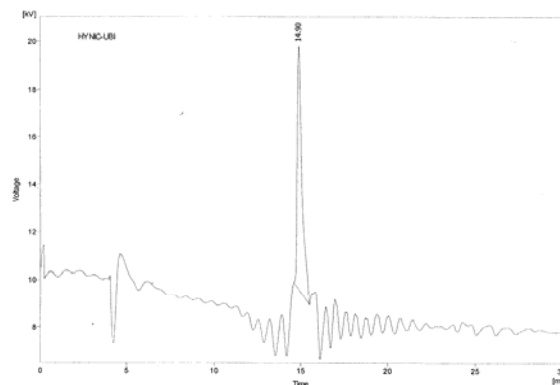


Fig. 2: RP-HPLC analysis of [HYNIC]-UBI 29-41

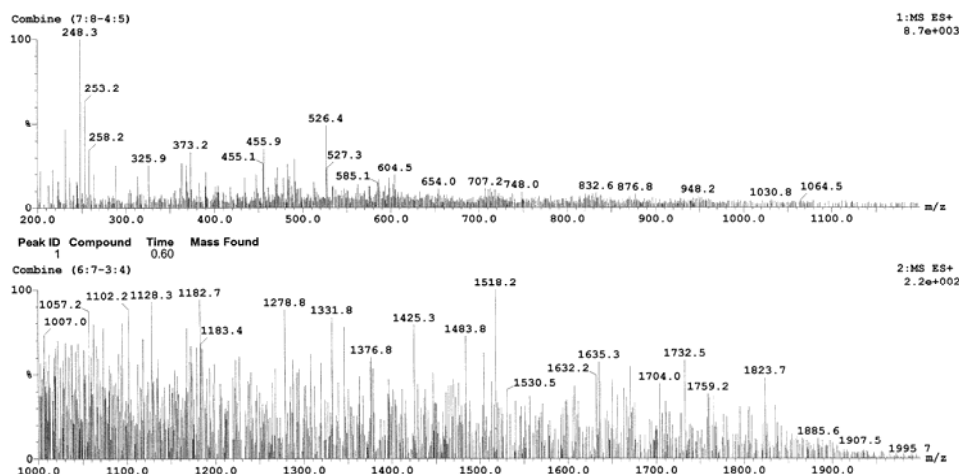


Fig. 3: Mass spectrometric analysis of [HYNIC]-UBI 29-41

Table 1: Biodistribution in mice with thigh muscle infection after injection of [^{99m}Tc-HYNIC]-UBI 29-41. Data are presented as %ID/g±SD and results are the means of groups of four animals.

Organ	Time		
	30 min	1 h	2 h
Blood	1.01 ± 0.07	0.91 ± 0.04	0.81 ± 0.02
Urine	1.39 ± 0.22	2.33 ± 0.23	3.11 ± 0.32
Kidney	35.69 ± 14.30	30.13 ± 10.20	22.06 ± 6.03
Gut	1.89 ± 0.92	1.68 ± 0.06	1.10 ± 0.01
Spleen	0.66 ± 0.07	0.78 ± 0.04	0.79 ± 0.02
Stomach	2.90 ± 0.39	1.66 ± 0.04	1.09 ± 0.01
Intestine	0.85 ± 0.22	0.60 ± 0.11	0.49 ± 0.02
Liver	1.44 ± 0.01	1.32 ± 0.01	1.15 ± 0.03
Lung	1.13 ± 0.20	1.11 ± 0.02	0.92 ± 0.02
Heart	0.76 ± 0.09	0.54 ± 0.05	0.24 ± 0.03
Infected muscle	0.93 ± 0.09*	1.05 ± 0.02*	0.71 ± 0.05*
Non-infected muscle	0.45 ± 0.01	0.51 ± 0.25	0.42 ± 0.02
Infection/non-infection ratio	2.099 ± 0.05	2.051 ± 0.14	1.69 ± 0.04

* $P < 0.05$ vs. Non-infected muscle.

The purity was >97%, as confirmed by the HPLC method. The labeling yield of [^{99m}Tc-HYNIC]-UBI 29-41 was >95%, acquired via HPLC and also ITLC at

a specific activity of 50 GBq/μmol that was stable for 24 hours. The HPLC elution times were 5.03 min for ^{99m}TcO₄⁻ and 15.54 min for ^{99m}Tc peptide (Fig. 4).

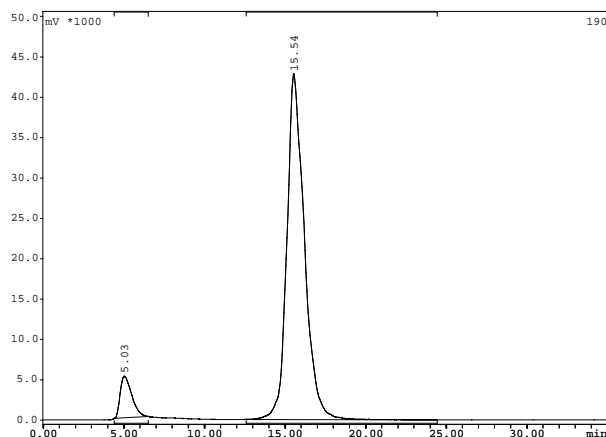


Fig. 4: RP-HPLC analysis of [^{99m}Tc -HYNIC]-UBI 29-41

Stability, affinity and binding assay

The affinity of the labeled peptide to human serum proteins after 24 hour was less than $25\pm 5\%$. Labeled peptide was stable in plasma with radiochemical purity of more than 90% after 24 h incubation. In vitro testing of [^{99m}Tc -HYNIC]-UBI 29-41 to *S. aureus* showed 75% of radioactivity bound to bacteria. In additional experiments we observed a significantly decrease by 60% in the binding to bacteria that had been pre-exposed for 1 h at 4°C with a 100 molar excess of [HYNIC]-UBI 29-41 peptide.

Animal biodistribution

The results of biodistribution in mice with *S. aureus* thigh muscle infection are summarized in Table 1. The radioligand displayed a rapid blood clearance mainly through the kidneys and subsequently the urinary bladder. Small amount of radioactivity were observed in the liver, spleen and thyroids. Accumulation of radiopeptide in the infected thigh muscles as indicated by a T/NT ratio was $2.099 \pm 0.05\%$ at 30 min after injection.

Typical Scintigrams of mice's with thigh muscle infection our inflammation and normal at 60 min after injection of radiopeptide are shown in Fig 5. High uptakes of activity in infection site with no accumulation in inflamed site were observed. Upon autopsy the bacterial infection had the same appearance as demonstrated on the scintigraphic image.

DISCUSSION

It should be realized that Ubiquicidin 29-41 can be labeled rapidly with high yields with ^{99m}Tc using a direct or indirect labeling technique (5-10).

Unfortunately, the mechanisms underlying the direct labeling of peptides with ^{99m}Tc are not well understood (5). This may be a drawback for the development of a kit based on the Ubiquicidin 29-41. Therefore, we considered the possibility of radiolabeling peptides through the intermediacy of a HYNIC as a chelator, prepared from lyophilized kit based on new formulation.

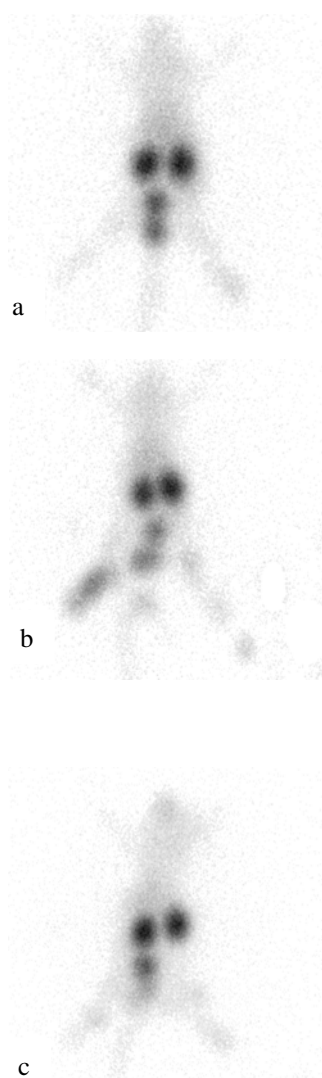


Fig. 5: Typical scintigrams of normal mice's (a), with thigh muscle infection (b) and inflammation (c) at 60 min after injection of [^{99m}Tc -HYNIC]-UBI 29-41.

The ^{99m}Tc labeling was straightforward for kit formulation with high radiochemical yields ($>95\%$)

and specific activities of about 50 GBq/μmol. The present results revealed that [^{99m}Tc-HYNIC]-UBI 29-41 preferentially bind to bacteria. It should be kept in mind that in our *in vitro* competition assays we observed inhibition of binding of [^{99m}Tc-HYNIC]-UBI 29-41 to *S. aureus* by unlabeled [HYNIC]-UBI 29-41 with maximal inhibition of approximately 60% when 100-fold excess of unlabeled peptide was used as competitor. Since the conditions of our *in vitro* competition assay do not represent the physiological texture at the site of infection, we cannot assume that a peptide would be successful at infection imaging on the basis of good *in vitro* binding to bacteria.

The biodistribution of radiopeptide showed a good correlation between *in vitro* binding and accumulation in site of infection in mice. [^{99m}Tc-HYNIC]-UBI 29-41 allow rapid visualization of infections with gram-positive and gram-negative bacteria with little or no accumulation in inflamed thigh muscles, indicating that these radiopeptide directly tag the bacteria at the site of infection.

Results from our new kit formulation were comparable with those obtained by Welling et al. [9, 10]. They used the same radiopharmaceutical but prepared from wet labeling method and different kit formulation, to study its biodistribution and observed binding of 40% and T/NT ratio of 1.84 ± 0.27 (%ID/g) after 2 h compared with 75% and 1.69 ± 0.04 (%ID/g) from our kit, respectively.

CONCLUSION

[^{99m}Tc-HYNIC]-UBI 29-41 obtained from lyophilized kit with 40 μg peptide can be used for differentiating infection compared with that of the sterile inflammatory site. The optimum time for imaging is 30 min after tracer injection.

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