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^{99m}Tc-HYNIC-Bombesin (7-14)NH_2: Radiochemical Evaluation with Co-ligands EDDA (EDDA = Ethylenediamine-N,N′-diacetic Acid), Tricine, and Nicotinic Acid

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Bombesin (BBN) is a peptide exhibiting high affinity for the gastrin releasing peptide receptor (GRPr), which is over-expressed on a variety of human tumors including breast, prostate, lung and pancreatic cancers. The specific aim of this study was to identify a new ^{99m}Tc-radiolabeled BBN analogue based upon the bifunctional chelating ligand HYNIC (2-hydrazinonicotinamide) that might be used as a noninvasive tool for diagnosis of GRP receptor-positive tumors. In this study, HYNIC-β-Ala-BBN(7-14)NH_2 and HYNIC-5-Ava-BBN(7-14)NH_2 were synthesized using traditional solid phase peptide synthetic techniques. The newly-formed conjugates were radiolabeled using ^{99m}Tc in the presence of different coligands including tricine, ethylenediamine diacetic acid (EDDA), tricine/EDDA, and tricine/nicotinic acid. Radiolabeling conditions (i.e., pH, temperature, and reaction time) were optimized and evaluated by ITLC and reversed-phase HPLC.

Keywords Bombesin, Technetium-99m, HYNIC, GRP

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INTRODUCTION

The design and development of diagnostic radiopharmaceuticals for early detection of human cancers has been investigated for several decades (Jurisson et al., 1993). Technetium-99m continues to be at the forefront of these investigations due to its ideal nuclear characteristics (6-hour half life and gamma energy of 140 keV), ready availability from a ^{99}Mo/^{99m}Tc generator, and well-established labeling chemistries (Jurisson et al., 1993).

In order to broaden the scope of radiopeptide scintigraphy, a wide spectrum of peptides have been proposed to target specific tumor cell types *in vitro* and *in vivo* (Smith et al., 2003). Bombesin (BBN), for example, is a tetradecapeptide, originally isolated from the skin of the amphibian *Bombina orientalis* (Lamberts et al., 1990). Bombesin has been found to have very high-affinity for gastrin releasing peptide (GRP) receptors. Furthermore, over-expression of receptors for

both BBN and GRP have been reported to be found on the cell surfaces of several malignant tissues (Jurisson et al., 1993), particularly in the cases of lung cancer (Karra et al., 1999a; Van de Wiele et al., 2001), colon cancer (Cuttitta et al., 1985), prostate cancer and breast cancer (Mahmoud et al., 1991; Preston et al., 1995). Therefore, bombesin analogues are considered a promising class of ligands toward the design and development of diagnostic/therapeutic, receptor-specific, radiolabelled peptides (Lamberts et al., 1990; Smith et al., 2003).

BBN analogues have been radiolabeled with a host of radionuclides including Copper-64 (Rogers et al., 2003), Rhodium-105 (Hoffman et al., 1997), Lutetium-177 (Smith et al., 2003b), Iodine-125 (Rogers et al., 1997), Rhenium-188 (Smith et al., 2003c) and Indium-111 (Hoffman et al., 2003). Recently, several new BBN conjugates radiolabeled with Tc(V)-99m have been reported as well (Alberto et al., 1999b; Baidoo et al., 1998; Karra et al., 1999b; La Bella et al., 2002; Schibli et al., 2002; Smith et al., 2002, 2003d; Zinn et al., 1998). Radiolabeling has been performed either directly or indirectly *via* a host of ligand frameworks including N_3S, P_2S_2, Dpr (Dpr = Diaminopropionic acid), and HYNIC (Alberto et al., 1999; Baidoo et al., 1998; Karra et al., 1999b; La Bella et al., 2002; Schibli et al., 2002; Smith et al., 2002, 2003d; Zinn et al., 1998). Schibli, Alberto, Schubiger, and Smith have recently employed an "organometallic" labeling strategy to produce site-directed ^{99m}Tc(I)-conjugates of BBN that specifically target human prostate cancer (Alberto et al., 1999; La Bella et al., 2002; Schibli et al., 2002; Smith et al., 2002; Zinn et al., 1998).

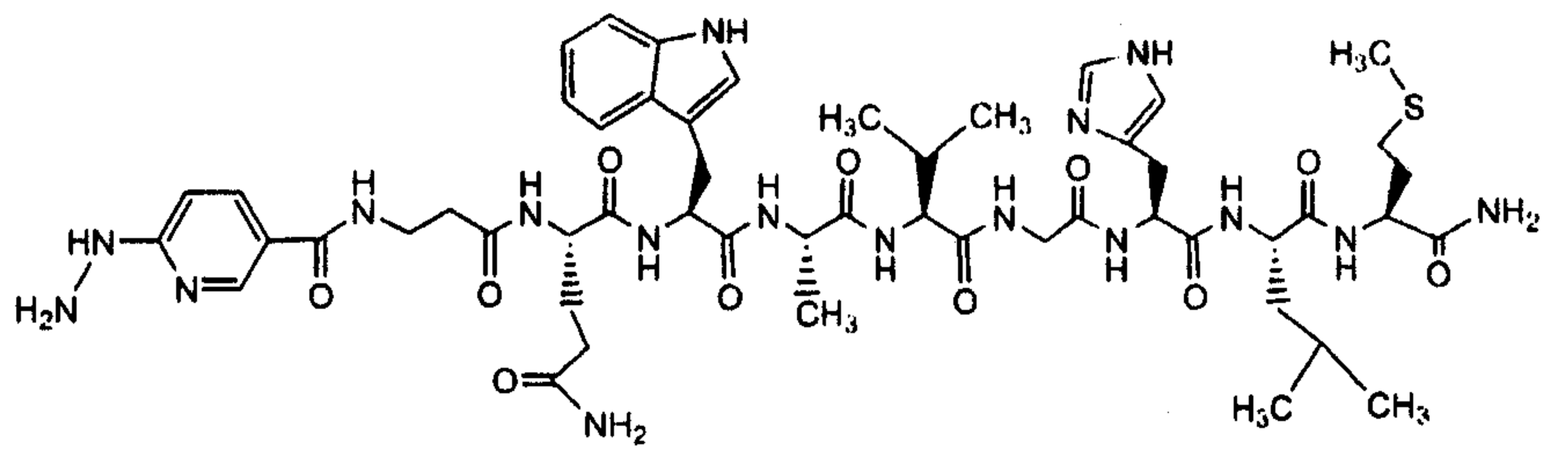
The search for novel conjugates for the development of *in vivo* stable, matched-pair ^{99m}Tc/^{188}Re-radiopharmaceuticals continues to be of significant interest. 2-hydrazinonicotinamide, HYNIC, is a bifunctional chelating ligand that has received considerable interest in recent years toward design and development of technetium- and rhenium-based radiopharmaceuticals. The use of the ^{99m}Tc-HYNIC core was first reported by Abrams et al. (Abrams et al., 1990), for the labeling of polyclonal IgG. Since then, HYNIC has been conjugated to various biomolecules including antibodies (Verbeke et al., 2003), chemotactic peptides (Babich and Fischman, 1995), somatostatin analogues (Decristoforo and Mather, 1999a), antisense-oligonucleotides (Hnatowich et al., 1995), interleukin-8 (Rennen et al., 2002) and many others (Decristoforo and Mather, 1999b; Guo, Hinkle and Lee, 1999). Zinn and co-workers have reported imaging of adenoviral gene transfer of GRP using a ^{99m}Tc-HYNIC-BBN conjugate (Zinn et al., 1998). In this study, he and his group synthesized a HYNIC-BBN derivative, with the HYNIC moiety directly conjugated to the N-terminal glutamine of BBN(7–14). The conjugate demonstrated specific, high-affinity binding for the GRP receptor, had optimal *in vivo* pharmacokinetic properties, and specifically targeted an adenoviral vector encoding GRPr in CD1 mouse models (Zinn et al., 1998).

Technetium-99m binds to the hydrazino-moiety forming a ^{99m}Tc-N bond (Decristoforo and Mather, 1999; Fichna, and Janecka, 2003). As HYNIC alone cannot satisfy the coordination requirements of Tc(V) (HYNIC can only occupy one or two coordination sites on the radionuclide), coligands are necessary to complete the coordination sphere of the technetium(V) core (Babish and Fischman, 1995). Several coligands have been developed to improve the ^{99m}Tc-radiolabeling of HYNIC-biomolecules. For example, glucoheptonate (Liu et al., 1996), tricine, and ethylenediamine diacetic acid (EDDA) (Decristoforo and Mather, 1999a, 1999b; Verbeke et al., 2003) have been used extensively toward the design and development of ^{99m}Tc-HYNIC radiopharmaceuticals. Liu and co-workers have described the use of ternary ligand systems to complete the coordination environment of the Tc(V)-HYNIC metal fragment. For example, they have used a water soluble phosphine or an imine-N-containing heterocycle as an additional coligand to form a ternary ligand framework (Liu et al., 1998, 1997). Other ternary ligand systems that have been reported include tricine/pyridine (Decristoforo and Mather, 1999a; Liu et al., 1998), tricine/nicotinic acid (Decristoforo and Mather, 1999a, 1999b; Rennen, 2002) and tricine/trisodium triphenylphosphine-3,3′,3″-trisulfonate (TPPTS) (Guo et al., 1999).

We herein report the synthesis and ^{99m}Tc-radiolabeling of HYNIC-β-Ala-BBN(7-14)NH_2 and HYNIC-5-Ava-BBN(7-14)NH_2 (Figure 1) using 2-hydrazinonicotinamide (HYNIC) as a bifunctional conjugating agent. The goal of this study was the development and assessment of specific radiolabeling conditions for the production of an *in vitro/in vivo* stable ^{99m}Tc-HYNIC-BBN conjugate. In this study, labeling of these novel conjugates with ^{99m}Tc was performed using different coligands [i.e., Tricine, EDDA, Tricine + Nicotinic Acid (Ternary Ligand Addition), and Tricine/EDDA Exchange Labeling] in order to assess the most optimum conditions for radiolabeling and usage in biological applications.

EXPERIMENTAL

6-BOC-hydrazinonicotinic acid (BOC-HYNIC) was purchased from SoluLink Biosciences, San Diego, California. Technetium-99m was eluted in ~6 milileters of isotonic saline solution from an alumina-based ^{99}Mo/^{99m}Tc generator, supplied locally by the Radiopharmacy Center of the Institute of Energetic and Nuclear Research (IPEN/CNEN)–Sao Paulo, Brazil. All other reagents were purchased from Sigma-Aldrich Brazil Ltda., and used without further purification. Peptide synthesis was performed on a Perkin-Elmer-Applied Biosystem Model 432 automated peptide synthesizer employing traditional Fmoc chemistry. The reaction of HBTU activated carboxyl groups on the reactant with the N-terminal amino group on the growing peptide, anchored *via* the C-terminus to the resin, provided for stepwise amino acid addition. Rink amide MBHA resin (25 μmole) and Fmoc-protected amino acids, with appropriate side-chain



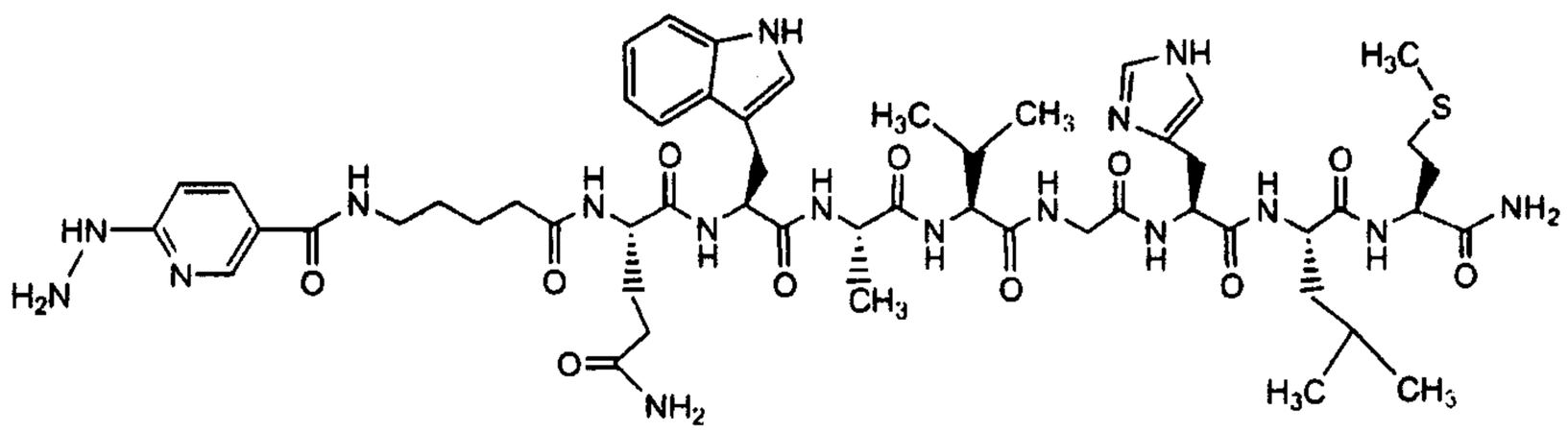


FIG. 1. Representative structures of HYNIC-β-Ala-BBN(7-14)NH_2 and HYNIC-5-Ava-BBN(7-14)NH_2.

protections, and the Fmoc-protected ω-$NH_2(CH_2)_nCOOH$ compounds used as spacer groups (75 μmol), were used for SPPS of the non-metallated BBN conjugates. The preselected synthetic sequence was designed to produce the HYNIC-BBN conjugates with the following general structure HYNIC-**X**-Q-W-A-V-G-H-L-M-NH_2, where the spacer group, **X** = ω-$NH_2(CH_2)_2COOH$ or ω-$NH_2(CH_2)_4COOH$, (See Figure 1 for structures of these HYNIC-**X**-BBN[7-14]NH_2 conjugates). The final products were cleaved by a standard procedure using a cocktail containing thioanisol, water, ethanedithiol and trifluoracetic acid in a ratio of 2 : 1 : 1 : 36 and precipitated into methyl-t-butyl ether. High performance liquid chromatographic (HPLC) analysis of the non-radiolabeled compounds was performed on a Waters 600E system equipped with a JASCO UV 975 tunable absorbance detector, an Eppendorf CH-30 column heater, an in-line EG&G ORTEC NaI solid scintillation detector, and a Hewlett Packard 3395 integrator. HPLC solvents were purchased from Fisher Scientific (Pittsburgh, PA) and used without further purification. Typical yields of the crude peptides were 80–85%. ES-MS was used in order to determine the molecular constitution of the HYNIC-**X**-BBN[7-14]NH_2 conjugates.

Tc-99m Radiolabeling of HYNIC-β-Ala-BBN(7-14)NH_2 and HYNIC-5-Ava-BBN(7-14)NH_2 (Figure 2)

Tris(hydroxymethyl-methylglycine (Tricine) as Co-ligand

To a sealed reaction vial was added 25 μg of either HYNIC-β-Ala-BBN(7-14)NH_2 or HYNIC-5-Ava-BBN(7-14)NH_2 and 40 mg of tricine co-ligand in 0.5 mL of 0.1 M phosphate buffer. This solution was purged with nitrogen for several minutes. To this solution was added 15 μL of 9 mM $SnCl_2H_2O$ solution in 0.1 N HCl (nitrogen-purged). Finally, 100 μL of $Na^{99m}TcO_4^-$ was added to the solution. The solution was heated for 15 minutes in a water bath at 100 °C and cooled to room temperature before further analyses were made. The pH of the reaction cocktail was 7.

Ethylenediaminediacetic Acid (EDDA) as Co-ligand

To a sealed reaction vial was added 25 μg of either HYNIC-β-Ala-BBN(7-14)NH_2 or HYNIC-5-Ava-BBN(7-14)NH_2 and 5 mg of EDDA co-ligand in 0.5 mL of 0.1 M phosphate buffer. This solution was purged with nitrogen for several minutes. To this solution was added 15 μL of 9 mM $SnCl_2 \cdot H_2O$ solution in 0.1 N HCl (nitrogen-purged). Finally, 100 μL of $Na^{99m}TcO_4^-$ was added to the solution. The solution was heated for 15 minutes in a water bath at 100 °C and cooled to room temperature before further analyses were made. The pH of the reaction cocktail was 7.

Tricine/EDDA Exchange Labeling

To a sealed reaction vial was added 25 μg of either HYNIC-β-Ala-BBN(7-14)NH_2 or HYNIC-5-Ava-BBN(7-14)NH_2, 20 mg of tricine, and 5 mg of EDDA in 0.5 mL of 0.1 M phosphate buffer. This solution was purged with nitrogen for several minutes. To this solution was added 15 μL of 9 mM $SnCl_2 \cdot H_2O$ solution in 0.1 N HCl (nitrogen-purged). Finally, 100 μL of $Na^{99m}TcO_4^-$ was added to the solution. The

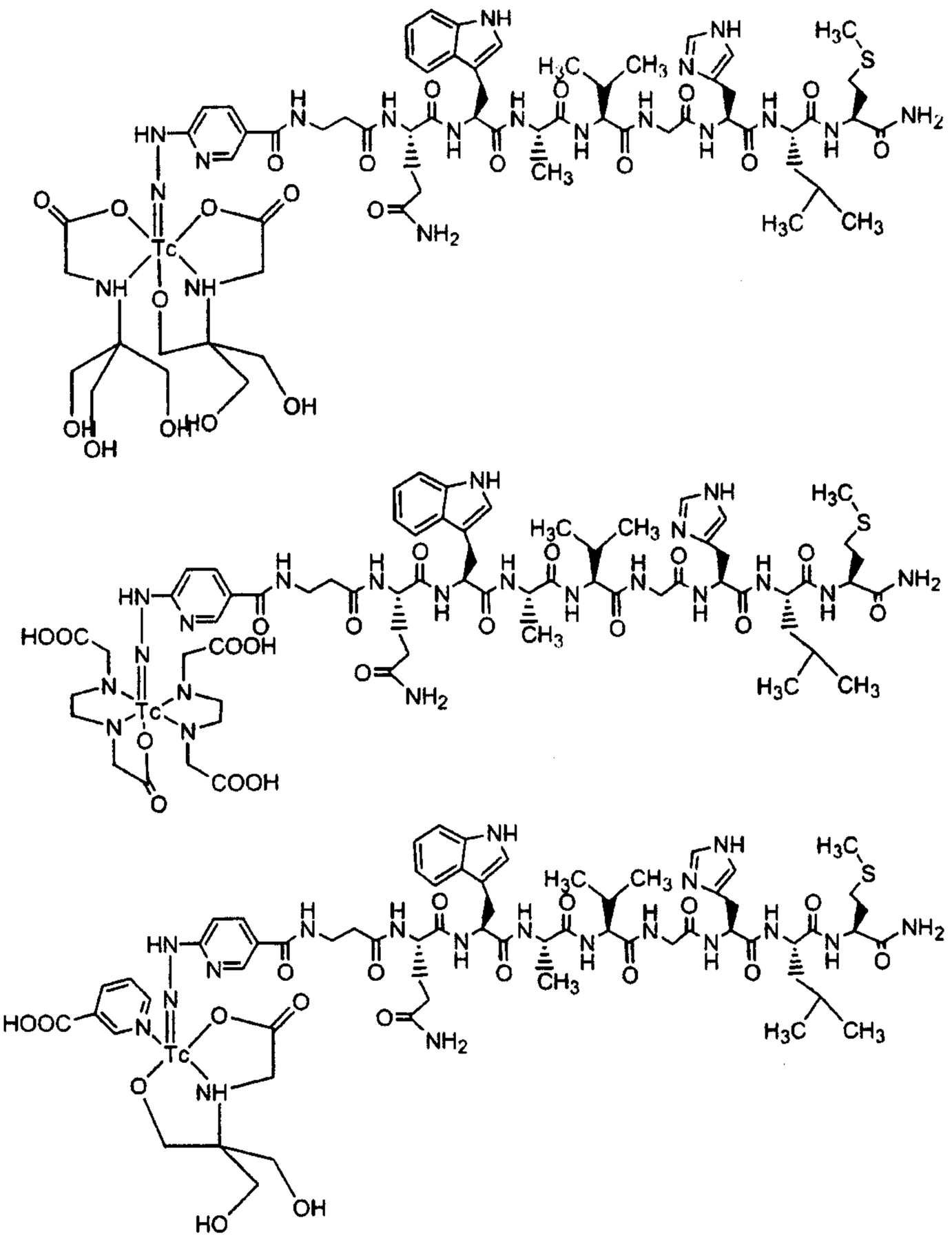


FIG. 2. Proposed structures of ^{99m}Tc-(Tricine)-HYNIC-β-Ala-BBN(7-14)NH_2 (1), ^{99m}Tc-(EDDA)-HYNIC-β-Ala-BBN(7-14)NH_2 (2), and ^{99m}Tc-(Tricine/Nicotinic Acid)-HYNIC-β-Ala-BBN(7-14)NH_2 (3).

solution was heated for 15 minutes in a water bath at 100 °C and cooled to room temperature before further analyses were made. The pH of the reaction cocktail was 7.

Tricine/Nicotinic Acid Ternary Ligands

To a sealed reaction vial was added 25 μg of either HYNIC-β-Ala-BBN(7-14)NH_2 or HYNIC-5-Ava-BBN(7-14)NH_2, 40 mg of tricine, and 2 mg of nicotinic acid in 0.5 mL of 0.1 M phosphate buffer. This solution was purged with nitrogen for several minutes. To this solution was added 10 μL of 9 mM $SnCl_2 \cdot H_2O$ solution in 0.1 N HCl (nitrogen-purged). Finally, 100 μL of $Na^{99m}TcO_4^-$ was added to the solution. The solution was heated for 15 minutes in a water bath at 100 °C and cooled to room temperature before further analyses were made. The pH of the reaction cocktail was 7.

Radiochemical Evaluation of the ^{99m}Tc-HYNIC-BBN Conjugates

Radiochemical analysis of the ^{99m}Tc-HYNIC-BBN conjugates was performed by instant thin-layer chromatography (ITLC) on silica gel strips (ITLC-SG, Gelman Sciences, Ann Arbor, Mich.) with a two solvent system (Methylethylketone (MEK) and 50% Acetonitrile (ACN)). In methylethylketone (MEK), $^{99m}TcO_4$- had an R_f of 1, $^{99m}TcO_2$ had an R_f of 0, and the radiolabeled conjugates had an R_f of 0. In 50% acetonitrile solution, $^{99m}TcO_4$- had an R_f of 0, $^{99m}TcO_2$ had an R_f of 0, and the radiolabeled conjugates had an R_f of 1. The ^{99m}Tc-HYNIC-BBN conjugates were also characterized by reverse phase high performance liquid chromatography (RP-HPLC). High performance liquid chromatographic (HPLC) analysis of the radiolabeled conjugates was performed on a Waters

600E system equipped with a Waters 486 tunable absorbance detector, an in-line Packard 150TR flow scintillation analyzer, and a Waters 746 data module. HPLC solvents consisted of H_2O containing 0.1% trifluoroacetic acid (Solvent A) and acetonitrile containing 0.1% trifluoracetic acid (Solvent B). A Symmetry C-18 column (5.0 μm, 100 Å, 4.6 × 250 mm, Waters, Milford, MA). C-18 (5 μm, 4.6 × 250 mm) was used with a flow rate of 0.5 ml/min. The HPLC gradient system begins with a solvent composition of 95%A and 5%B and follows a linear gradient of 30%A : 70%B from 0–25 min, and 30%A : 70%B to 5%A:95%B from 25–30 min.

RESULTS

The introduction of a spacer into a biomolecular targeting vector for attachment of the radiolabel is considered to reduce interferences with the receptor binding sequences of that conjugate. In this study, The HYNIC-X-BBN(7-14)NH_2 (Figure 1) conjugates were conveniently synthesized by SPPS. The yields of the HPLC purified conjugates were approximately 50%. ES-MS analyses were consistent with the molecular weights calculated for each conjugate (HYNIC-β-Ala-BBN(7-14)NH_2; Calculated: 1146.3, Found: 1146.8. HYNIC-5-Ava-BBN(7-14)NH_2; Calculated: 1174.4, Found: 1174.7).

The Tc-99m HYNIC β-Ala and 5-Ava conjugates of BBN(7-14)NH2 (Figure 2) were prepared by methods similar to those previously reported (von Guggenberg et al., 2004). Briefly, the HYNIC-conjugates were incubated with co-ligand, $^{99m}TcO_4^-$, and reducing agent in a boiling water bath for a period of 15 minutes. ^{99m}Tc-HYNIC-β-Ala-BBN(7-14)NH_2 and ^{99m}Tc-HYNIC-5-Ava-BBN(7-14)NH_2 conjugates with different coordinating co-ligands were produced in high radiochemical yield (≥90%) under these conditions. The presence of $^{99m}TcO_4^-$ and of $^{99m}TcO_2$ in the reaction mixtures was evaluated by ITLC on silica gel gel strips using Methylethylketone (MEK) and 50% Acetonitrile (ACN) as eluents. In methylethylketone (MEK), $^{99m}TcO_4^-$ had an R_f of 1, $^{99m}TcO_2$ had an R_f of 0, and the radiolabeled conjugates had an R_f of 0. In 50% acetonitrile solution, $^{99m}TcO_4$- had an R_f of 0, $^{99m}TcO_2$ had an R_f of 0, and the radiolabeled conjugates had an R_f of 1. These results can be viewed in Tables 1 and 2, respectively. We observed very little difference in the amounts of $^{99m}TcO_2$, $^{99m}TcO_4^-$, and labeled conjugate for all of the co-ligands used. However, when EDDA was used as a stand-alone co-ligand, more free $^{99m}TcO_4^-$ was observed in the reaction mixture (Tables 1 and 2). The radiochemical yield obtained for the exchange radiolabeling system, HYNIC-BBN + tricine +EDDA was ≥98%. For the ternary ligand system of HYNIC-BBN/tricine/nicotinic acid, radiolabeling yields were ~99 ± 0.1%. $^{99m}TcO_4^-$ and $^{99m}TcO_2$ impurities in the crude reaction mixture were less than 2%. As a control radiolabeling of the co-ligands was also performed in the absence of the HYNIC-BBN conjugates. Reaction conditions were identical to those reported above.

TABLE 1
Radiochemical purity evaluation of ^{99m}Tc-HYNIC-β-Ala-BBN(7-14)NH_2 using different coligands as evaluated by ITLC (n = 8)

Coligands	$^{99m}TcO_4^-$	$^{99m}TcO_2$	^{99m}Tc-Ala HYNIC-BBN
Tricine	2.71 ± 0.43	3.36 ± 0.62	93.93 ± 1.05
EDDA	5.24 ± 0.82	2.12 ± 0.74	92.64 ± 1.56
Tricine + EDDA	0.23 ± 0.10	0.87 ± 0.21	98.90 ± 0.31
Tricine + nicotinic acid	0.06 ± 0.01	0.57 ± 0.10	99.37 ± 0.11

In order to optimize radiochemical yields, different ligand (ranging from 10 μg to 50 μg) and stannous ion concentrations were used during the radiolabeling protocols. For these investigations, there was essentially no difference in labeling yields when the mass of conjugate was varied between 10 μg and 50 μg. Furthermore, stannous ion concentrations above 10 μg showed no appreciable increase in amount of ^{99m}Tc colloid in the reaction mixture.

The radiochemical purity of the new conjugates was also evaluated by RP-HPLC. For all of the crude reaction mixtures $^{99m}TcO_4^-$ and radiolabeled conjugate were observed as major species. The crude HPLC chromatographic profiles for the ^{99m}Tc-EDDA-HYNIC-BBN conjugates produced *via* tricine transmetallation are shown in Figure 3. The chromatograms show a single peak for each the ^{99m}Tc-EDDA-HYNIC-β-Ala-BBN(7-14)NH_2 and ^{99m}Tc-EDDA-HYNIC-5-Ava-BBN(7-14)NH_2 conjugates with retention times of 14.9 and 15.2 min, respectively. Pertechnetate had a retention time of 5.3 min under these HPLC conditions (Figure 3). These products had a very good *in vitro* stability profile of ≥24 h. For example, the Tc-99m HYNIC β-Ala and 5-Ava conjugates of BBN(7-14)NH2 were stable at normal pH (~7), showing intact complexes with yields of 97.14 ± 0.79% and

TABLE 2
Radiochemical purity evaluation of ^{99m}Tc-HYNIC-5-Ava-BBN(7-14)NH_2 using different coligands as evaluated by ITLC (n = 8)

Coligands	$^{99m}TcO_4^-$	$^{99m}TcO_2$	^{99m}Tc-Ala HYNIC-BBN
Tricine	1.76 ± 0.31	1.47 ± 0.38	96.77 ± 0.69
EDDA	4.52 ± 0.57	1.77 ± 0.24	93.71 ± 0.81
Tricine + EDDA	0.64 ± 0.14	0.44 ± 0.11	98.92 ± 0.25
Tricine + nicotinic acid	0.08 ± 0.02	0.37 ± 0.07	99.55 ± 0.09

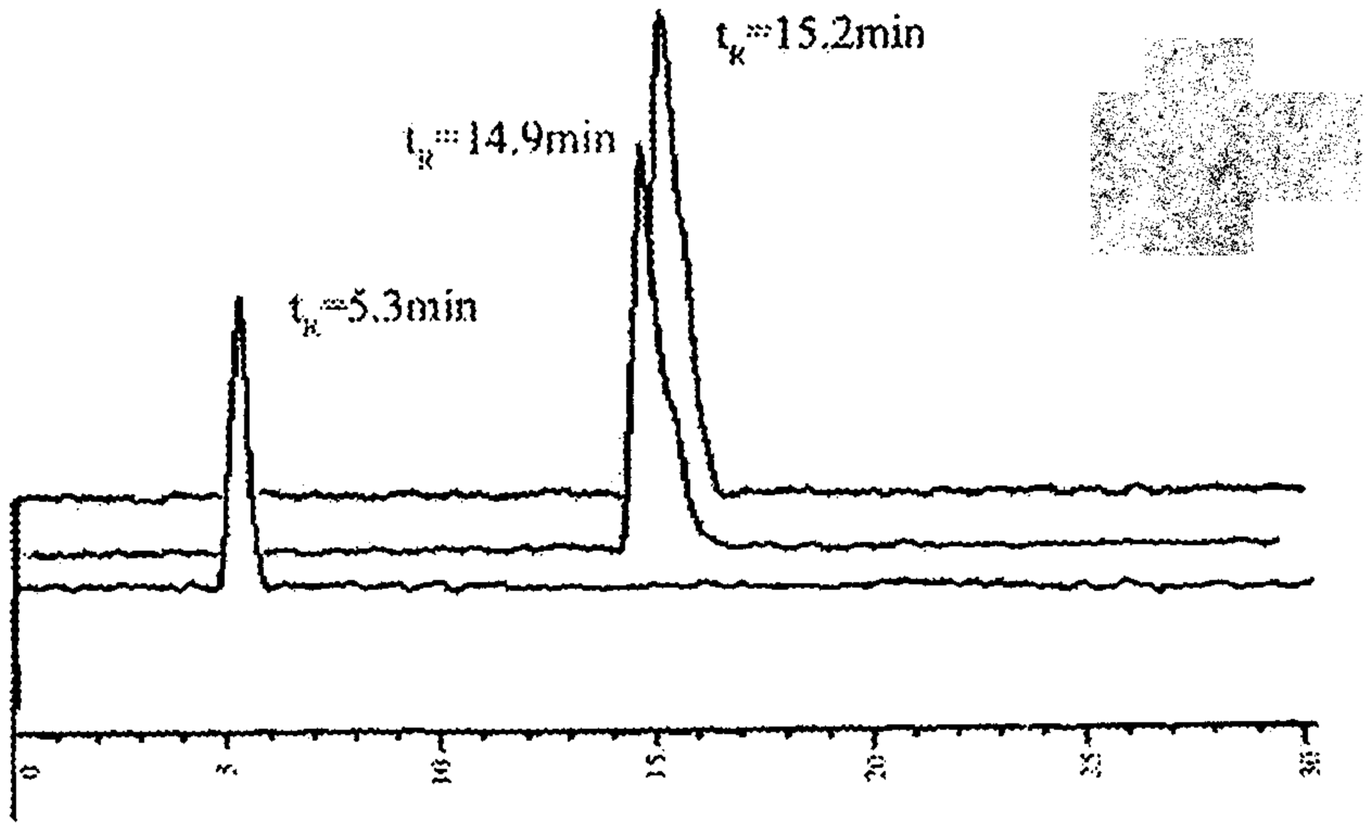


FIG. 3. Reversed-phase chromatographic profile of $^{99m}TcO_4^-$, ^{99m}Tc-HYNIC-β-Ala-BBN(7-14)NH_2, and ^{99m}Tc-HYNIC-5-Ava-BBN(7-14)NH_2. retention times of 5.3, 14.9, and 15.2, respectively.

96.97 $\pm$ 1.05%, respectively. The specific activity for these conjugates was ~6250 MBq/μmol.

DISCUSSION

Central to the development of site-directed radiopharmaceuticals is the search to find a specific ligand system that is easily conjugated to a targeting vector and radiolabeled in high yield in a very short amount of time. Furthermore, it is a necessity that the metallic radionuclide be stabilized under *in vivo* conditions to the likes of serum-based proteins such as transferrin. Hydrazinonicotinic acid (HYNIC) is a bifunctional conjugating ligand that has been used for ^{99m}Tc-radiolabeling of a number of peptides including bombesin (Babish and Fischman, 1995; Decristoforo and Mather, 1999a; Zinn et al., 1998). 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 (Babish and Fischman, 1995; Decristoforo and Mather, 1999a; Rennen et al., 2002). The use of coligands allows for easy modification of the hydrophilicity and pharmacokinetics of ^{99m}Tc-labeled peptide conjugates. In this investigation we have examined the influence of different coligands in the Tc-99m radiolabeling procedures for two HYNIC-BBN derivatives in order to produce an *in vitro/in vivo* stable ^{99m}Tc-HYNIC-BBN conjugate.

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. For most of these studies, concentration of tricine has been a critical issue, influencing the radiochemical purity of the final product. In these studies, tricine concentrations have varied dramatically. For example, quantities of 15 to 50 mg (83.7 to 279 μmol) have been used in the radiolabeling cocktails (Bangard et al., 2000; Guo et al., 1999; Rennen et al., 2000). Liu and Edwards (Liu and Edwards, 1999) have reported that the use of tricine or glucoheptonate as coligands suffers from the instability of technetium complexes in the absence of excess coligand, and the presence of multiple species of these complexes in solution, due to different bonding modalities of HYNIC and the tricine or glucoheptonate coligands (Edwards et al., 1997). 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 (Edwards et al., 1997). This observation has also been reported by Su and co-workers (Su et al., 2003). In their study, they reported that a reduced amount of tricine in the ^{99m}Tc-labeling procedure of HYNIC-RGD resulted in ~10 – 15% ^{99m}Tc-radiocolloid ($^{99m}TcO_2$) (Su et al., 2003). Unreacted $^{99m}TcO_4$- was on the order of 3–5% in this particular study (Su et al., 2003). In this study we have made an attempt to avert those problems previously reported in the literature by using a greater concentration of tricine (80 mg/mL) at a pH of 7. When tricine was used as a stand-alone coligand, the formation of radiocolloid (3.36 $\pm$ 0.62% and 1.47 $\pm$ 0.38% for the β-Ala and 5-Ava derivatives, respectively) was only minimally present as indicated by ITLC and is an improvement over other literature preparations. Radiochemical yields for the new ^{99m}Tc-HYNIC-BBN conjugates, HYNIC-β-Ala-BBN(7-14)NH_2 and HYNIC-5-Ava-BBN(7-14)NH_2, when tricine was used as a stand-alone co-ligand, were 93.93 $\pm$ 1.05 and 96.77 $\pm$ 0.69%, respectively. Non-reacted $^{99m}TcO_4^-$ (^{99m}Tc-HYNIC-β-Ala-BBN(7-14)NH_2, 2.71 $\pm$ 0.43%; ^{99m}Tc-HYNIC-5-Ava-BBN(7-14)NH_2, 1.76 $\pm$ 0.31%) was also present in the reaction mixture as indicated by ITLC (Tables 1 and 2). Quality control by ITLC was an effective way of determining the presence of ^{99m}Tc- pertechnetate and ^{99m}Tc-radiocolloid in this study. However, this method does not make clear if

there are different isomeric forms of the new ^{99m}Tc-HYNIC-conjugates. For the new derivatives of ^{99m}Tc-Tricine-HYNIC-β-Ala-BBN(7-14)NH_2 and ^{99m}Tc-Tricine-HYNIC-5-Ava-BBN(7-14)NH_2, RP-HPLC indicated a single major species with some minor species also present in the chromatogram (data not shown). Liu and co-workers have reported that HPLC analysis of ^{99m}Tc-HYNIC-conjugates with acetonitrile as an eluting solvent may generate mixed tricine/acetonitrile coligand complexes on the column that are not present in the sample under analysis (Liu et al., 2002). This may explain the presence of small impurities observed in our reversed-phase chromatograms when tricine was used in the reaction mixture.

The coligand EDDA is also of particular interest because it is a potentially tetradentate ligand and is expected to form a more symmetrical and stable complex with technetium when compared to tricine. Ideally, the higher symmetry should result in fewer coordination isomers than when tricine is used as a coligand (Liu et al., 1996, 1997). For our study, we found no appreciable difference in the radiochemical yields when comparing the coligands tricine and EDDA to one another. Radiochemical yields for the new ^{99m}Tc-HYNIC-BBN conjugates, HYNIC-β-Ala-BBN(7-14)NH_2 and HYNIC-5-Ava-BBN(7-14)NH_2, when EDDA was used as a stand-alone co-ligand, were 93.93 $\pm$ 1.05 and 96.77 $\pm$ 0.69%, respectively. The formation of $^{99m}TcO_2$ (2.12 $\pm$ 0.74% (β-Ala) and 1.77 $\pm$ 0.24% (5-Ava)) was also observed. Non-reacted $^{99m}TcO_4$- (^{99m}Tc-HYNIC-β-Ala-BBN(7-14)NH_2, 5.24 $\pm$ 0.82%; ^{99m}Tc-HYNIC-5-Ava-BBN(7-14)NH_2, 4.52 $\pm$ 0.57%) was also present in the reaction mixture as indicated by ITLC. These reports are very similar to those of Su et al., who also compared the unbound ^{99m}Tc in the labeling of RGD-HYNIC using tricine and EDDA as coligands (Su et al., 2003). In the case of tricine being used as a coligand, they obtained 3–5% $^{99m}TcO_4^-$ in their reaction mixtures. When EDDA was used as a co-ligand, a larger percentage of this impurity was revealed.

Early studies of Decristoforo and co-workers (Decristoforo et al., 2000), employed either tricine or EDDA as coligands in the radiolabeling of Tyr^3-octreotide (TOC) with ^{99m}Tc. However, clinical reports by Plachcinska et al., and Gabriel et al., have indicated that the use of using both coligands together to produce ^{99m}Tc-HYNIC-TOC *via* a trans-metallation type of reaction, produced very good results (Gabriel et al., 2004; Plachcinska et al., 2003). Decristoforo has recently reported this exchange type of labeling to be very effective in producing ^{99m}Tc-conjugates of [D-Glu^1]-Minigastrin (von Guggenberg et al., 2004). In this study, we saw a dramatic increase in radiolabeling yield when employing the exchange labeling technology *via* tricine and EDDA. For example, ^{99m}Tc-EDDA-HYNIC-β-Ala-BBN(7-14)NH_2 and ^{99m}Tc-EDDA-HYNIC-5-Ava-BBN(7-14)NH_2 (98.90 $\pm$ 0.31 and 98.92 $\pm$ 0.25%, respectively) were produced in very high yields when tricine was used as a trans ligating moiety. Furthermore, the presence of $^{99m}TcO_4$ and reduced hydrolyzed technetium by-product was greatly diminished using this reaction procedure (Tables 1 and 2). The crude HPLC chromatographic profiles for the ^{99m}Tc-EDDA-HYNIC-BBN conjugates produced *via* tricine transmetallation are shown in Figure 3. The chromatograms show a single peak for each the ^{99m}Tc-EDDA-HYNIC-β-Ala-BBN(7-14)NH_2 and ^{99m}Tc-EDDA-HYNIC-5-Ava-BBN(7-14)NH_2 conjugates with retention times of 14.9 and 15.2 min, respectively. It should be pointed out that the more hydrophilic compound, ^{99m}Tc-EDDA-HYNIC-β-Ala-BBN(7-14)NH_2, is retained to a lesser extent on the C-18 reversed-phase HPLC column.

The use of ternary complexes of ^{99m}Tc-HYNIC has been reported. Conjugates of this type add the advantage of high *in vivo* conjugate stability (Decristoforo and Mather, 1999; Liu et al., 1998, 1997). For example, Liu et al., have reported the use of a ternary-system using tricine and water-soluble phosphines as coligands. The new conjugates were produced in very high yield and high specific activity, in two isomeric forms (Liu et al., 1996; Liu and Edwards, 1999). In our study we used tricine and nicotinic acid to form a ternary ligand system about the 5+ metal center of ^{99m}Tc. We could not observe the presence of isomeric formation *via* ITLC, and only minor impurities were indicated by RP-HPLC (data not shown). Radiochemical yields for the new ^{99m}Tc-HYNIC-BBN conjugates, HYNIC-β-Ala-BBN(7-14)NH_2 and HYNIC-5-Ava-BBN(7-14)NH_2, when tricine/nicotinic acid were used to form the ternary ligand system, were 99.37 $\pm$ 0.11 and 99.55 $\pm$ 0.09% respectively. $^{99m}TcO_4^-$ and $^{99m}TcO_2$ were also present in minor amounts as indicated by ITLC (Tables 1 and 2).

CONCLUSION

In this study, we have shown multiple synthetic approaches toward ^{99m}Tc-radiolabeling of HYNIC-β-Ala-BBN(7-14)NH_2 and HYNIC-5-Ava-BBN(7-14)NH_2. In this study, labeling of these novel conjugates with ^{99m}Tc was performed using different coligands (i.e., Tricine, EDDA, Tricine + Nicotinic Acid (Ternary Ligand Addition), and Tricine/EDDA Exchange Labeling) in order to assess the most optimum conditions for radiolabeling and potential usage in clincal applications. Using all of the radiolabeling conditions/co-ligands demonstrated herein, ^{99m}Tc-HYNIC-β-Ala-BBN(7-14)NH_2 and ^{99m}Tc-HYNIC-5-Ava-BBN(7-14)NH_2 were produced as a single species in high radiochemical yield. Reaction times were very short and facile making them ideal for usage in a clinical capacity by nuclear medicine technologists. Furthermore, those conjugates prepared by tricine/EDDA exchange labeling showed relatively high specific activity and demonstrated excellent radiochemical stability even out to 24 hours post incubation. These properties suggest that the ^{99m}Tc-HYNIC-BBN conjugates demonstrated herein hold some potential as site-directed diagnostic radiopharmaceuticals,

necessitating *in vivo* studies in human-cancer models. Those studies are currently underway.

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