Synthesis and Evaluation of a New Radiolabeled Bombesin Analogue for Diagnosis of GRP Receptor Expressing Tumors

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ABSTRACT

Introduction: Bombesin (BN), a 14-amino acid neuropeptide, shows high affinity for the human GRP (gastrin releasing peptide) receptors, which are overexpressed by a variety of cancers, including prostate, breast, pancreas, gastrointestinal, and small cell lung cancer. Aim was to prepare [6-hydrazinopyridine-3-carboxylic acid ($HYNIC^0$), $D\text{-}Tyr^6$, $D\text{-}Trp^8$] - BN [6-14] NH_2 that could be easily labeled with ^{99m}Tc and evaluation of its potential as an imaging agent.

Methods: Synthesis of the peptide amide was carried out onto Rink Amide MBHA (4-Methylbenzhydrylamine) resin. A bifunctional chelating agent (BFCA) was attached to the N terminal peptide in solid-phase. ^{99m}Tc labeling was performed by addition of sodium pertechnetate to solution that include [$HYNIC^0$, $D\text{-}Tyr^6$, $D\text{-}Trp^8$] Bombesin [6-14] NH_2, tricine, ethylenediamine-N,N′-diacetic acid (EDDA) and $SnCl_2$. Radiochemical evaluation was carried out by reverse phase high-performance liquid chromatography (HPLC) and instant thin layer chromatography (ITLC). In-vitro internalization was tested using human prostate cancer cells (PC-3) with blocked and non-blocked receptors. Biodistribution was determined in rats.

Results: [^{99m}Tc/tricine/EDDA-$HYNIC^0$, $D\text{-}Tyr^6$, $D\text{-}Trp^8$] bombesin [6-14] NH_2 was obtained with radiochemical purities >98%. Results of in-vitro studies demonstrated a high stability in serum and suitable internalization. Biodistribution data showed a rapid blood clearance, with renal excretion and specific binding towards GRP receptor-positive tissues such as pancreas.

Conclusion: In this study, labeling of this novel conjugate with ^{99m}Tc easily was performed using coligand. The prepared ^{99m}Tc-HYNIC-BN conjugate has promising characteristics for the diagnosis of malignant tumors.

Key words: Bombesin, ^{99m}Tc, Tumor, HYNIC

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INTRODUCTION

Bombesin is a tetradecapeptide, originally isolated from the skin of the amphibian *Bambino orientalis* (1). Bombesin has been found to have very high-affinity for GRP receptors (2). Furthermore, over-expression of receptors for both BN and GRP have been reported to be found on the cell surfaces of several malignant tissues, particularly in the cases of lung cancer, colon cancer, prostate cancer and breast cancer (3, 4). Many types of labeled BN analogues have been developed for evaluation of GRP receptor expressing tumors (5, 6). It has been shown that 7-14 amino acid sequence of BN with amidated c-terminus is necessary for receptor binding affinity and n-terminal of peptide can be used for labeling (7). Therefore, most radiolabeled BN analogues are based on 7–14 amino acid sequence, coupled with a chelator through a spacer group at the N-terminus of the peptide (8, 9). For example different conjugates were developed using bifunctional chelatores for ^{99m}Tc labeling, such as N_3S (triamidethiol) (10, 11), N_4 (tetraamine) (12, 13), HYNIC (14, 15), and carbonyl (16). Some ^{99m}Tc-labeled peptides are currently being investigated in gastrin releasing peptide receptor positive tumors in patients (17, 18).

Recently a synthetic analog of BN/GRP, (D-Tyr6, b-Ala11, Phe13, Nle14-NH2) BN [6-14], which possess high affinity to each of the three classes of mammalian BN/GRP receptors and functions as a universal ligand for all the three mammalian BN/GRP receptors, has been developed (19). In all works done in this topic, the main focus was on the type of chelate, spacer group and introduction of other amino acids, which may influence the binding affinity and pharmacokinetics of conjugate. To create a new ^{99m}Tc-labelled peptide for tumor targeting we choosed a BN [7-14] and a D-Tyr^6 as a spacer based on the above universal ligand also in order to improve excretion pattern via kidney. We also modified D-Trp^8 versus L-Trp^8 to decrease enzymatic metabolism (20).

We herein report the synthesis and ^{99m}Tc-radiolabeling of [HYNIC^0, D-Tyr^6, D-Trp^8] - Bombesin [6-14] NH_2 using coligand Tricine/EDDA. In addition we studied stability in human serum, internalization in PC-3 cells and the in vivo biodistribution of the radiolabeled peptide in rat.

METHODS

All chemicals were obtained from commercial sources and used without additional purification. Rink Amide MBHA resin and all of the Fmoc-protected amino acids were commercially available from NovaBiochem (Laufelfingen, Switzerland). The prochelator HYNIC-Boc was synthesized according to Abrams et al (21). Sodium pertechnetate ($Na^{99m}TcO_4$) was obtained from commercial ^{99}Mo/^{99m}Tc Generator (Radioisotope Division, AEOI). PC-3 cell line was obtained from Pasteur Institute of Iran and RPMI medium from Gibco®.

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. CC250/4.6 Nucleosil 120-5C18 column from Teknokroma was used for analytical HPLC, and a VP250/10 Nucleosil 100-5 C18 column was used for semipreparative HPLC. The gradient systems consisted of 0.1% trifluoroacetic acid/water (Solvent A) and acetonitrile (Solvent B). For analytic HPLC, Gradient I was used: 0 min 95% A (5% B), 5 min95% A (5% B), 20min 0% A (100% B), 23 min 0% A (100% B), 25 min 95% A (5% B), 35 min 95% A (5% B) flow=1 ml/min, λ=280 nm ; for semipreparative HPLC Gradient II: 0 min 80% A (20% B), 2 min 80% A (20% B), 17 min 50% A (50% B), 19 min 0%A (100% B), 21 min 0% A (100% B), 24 min 80%A (20% B), 32 min 80% A (20% B), flow=2

ml/min, λ=280 nm. Liquid Chromatography /Mass Spectrometry (LC/MS) and ^{1}H NMR (nuclear magnetic resonance) were carried out to determine the molecular constitution and to identify of the [HYNIC^{0}, D-Tyr^{6}, D-Trp^{8}] Bombesin [6-14] NH_2 conjugate.

Synthesis

Synthesis of the peptide amide was carried out by using *a*-fluorenylmethoxycarbonyl (Fmoc) amino acids, onto Rink Amide resin (substitution, 0. 69 mmol/g) following the method of Fmoc solid phase peptide synthesis. Coupling of each amino acid was performed in the presence of 3 mol excess of Fmoc–amino acid, 3 mol excess of N-hydroxybenzotriazole (HOBT), 3 mol excess of Diisopropylcarbodiimide (DIC) and 5 mol excess of diisopropyletylamine (DIPEA) in Dimethylformamide (DMF). Coupling success was checked by the established 2, 4, 6-trinitrobenzenesulfonicacid (TNBS) test. After suspending a few resin in DMF one drop of solution containing 10% DIPEA and 1% TNBS in DMF was added. A positive test was indicated by red beads (22). Removing of Fmoc group was achieved by repetitive treatment with 20% piperidine in DMF.

Coupling of HYNIC to peptide was performed in the presence of 1.2 mol excess of HYNIC-BOC 2.5 mol excess of (2-(7-Aza-1H-benzotriazole-1-yl)-1, 1, 3, 3-tetramethyluronium hexafluorophosphate) (HATU), 5 mol excess of diisopropyletylamine (DIPEA) in Dimethylformamide (DMF). Coupling success was checked by TNBS test. The peptide HYNIC conjugate was removed from the resin and amino acid side chains were deprotected by treatment with a cocktail of trifluoro acetic acid (TFA), water and triisopropylsilan. After removing the organic solvents, the crude product was precipitated with cold diethyl ether.

Analyses of peptide HYNIC conjugate

The crude peptide HYNIC conjugate was dissolved in water and purified by semi-preparative (Gradient II) RP-HPLC; next the purified product was characterized by LC/MS, ^{1}H NMR and UV. ^{1}H NMR spectrum was obtained on bruker 500MHz NMR spectrometer using D_2O as a solvent. Mass spectrum was recorded on an Agilent 1100/ Bruker Daltonic (Ion trap) VL instrument (LC/MS).

Labeling and Radiochemical Evaluation

20 µg of [HYNIC^{0}, D-Tyr^{6}, D-Trp^{8}] Bombesin [6-14] NH_2, 15 mg of tricine, and 5 mg of EDDA were added to a vial containing 0.5 mL of water. 40 µg $SnCl_2$ (20 µl of 2 mg/ml $SnCl_2$, $2H_2O$ in nitrogen-purged 0.1 M HCl) was added to vial. Finally, 0.5 mL of 370-1300 MBq of $Na^{99m}TcO_4$ in saline was added to the solution. The vial was heated for 10 minutes in a water bath at 100 ˚C and cooled to room temperature. After cooling to room temperature, the labeled peptide was analyzed by analytical RP-HPLC (Gradient I) and ITLC on silica gel 60 (Merck) using different mobile phases: 2-butanone for free $^{99m}TcO_4$ (R_f =1), 0.1 M sodium citrate (pH 5) to determine the non-peptidebound ^{99m}Tc coligand with $^{99m}TcO_4$ (R_f =1) and methanol/1M ammonium acetate 1/1 for ^{99m}Tc colloid (R_f =0).

Serum stability

A volume of 50 µl of the labelled peptide solution was incubated at 37˚C with 1ml of fresh human serum. Radiochemical stability was determined taking samples of 10 µl at different times to 24 h for analysis by ITLC.

Internalization

PC-3 cells (1×10^{6} per well in 6-well plates) supplied with fresh medium were washed once with 2 ml of internalization medium (RPMI with %1 FBS). Furthermore, 1.5 ml internalization medium was added to each well, and the plates were incubated at 37°C for about 1 h. Afterward, about 150 kBq (2.5

pmol total peptide mass per well) was added to the medium, and the cells were incubated at 37°C for various time periods. To determine nonspecific membrane binding and internalization, we incubated cells with the radioligand in the presence of 150 µl, 1 µmol cold peptide. The cellular uptake was stopped at appropriate time periods (1 h, 2 h and 4 h) by removing medium from the cells and washing twice with 1 ml of ice-cold phosphate-buffered saline (PBS). An acid wash for 10 min with a glycine buffer (pH 2.8) on ice was also performed twice. This step was to distinguish between membrane-bound (acid releasable) and internalized (acid resistant) radioligand. Finally, the cells were treated with 1 N NaOH. The culture medium and the receptor-bound and internalized fractions were measured radiometrically in a gamma counter.

Biodistribution

Animal experiments were performed in compliance with the regulations of nuclear science & technology research institute and with generally accepted guidelines governing such work. Six rats were injected with 20 MBq (0.35 nmol) ^{99m}Tc-peptide diluted in saline (total injected volume = 150 µL) into the femoral vein. In order to determine the non-specific uptake of the radiopeptides, in receptor-positive organs, a group of 3 animals were injected with 100 µg cold peptide in 50 µL saline as a co-injection with the radipeptides (blocked animals). After 4 h, the rats in groups of 3 animals were scarified, organs of interest were collected, weighed and radioactivity was measured in a gamma-counter. The percentage of the injected dose per gram (%ID/g) was calculated for each tissue.

Statistical analyses

The calculations of means and standard deviations for internalization and biodistribution were performed on Microsoft Excel. Student's *t* test was used to determine statistical significance. Differences at the 95% confidence level ($p<0.05$) were considered significant.

RESULTS AND DISCUSSION

The peptide HYNIC conjugate was prepared by Fmoc solid phase synthesis with an overall yield of 48% (Fig. 1). It was purified by RP-HPLC, yielding a highly pure final product, as characterized by analytical RP-HPLC as a single peak in retention time of 18.47 min (Fig. 2).

^{1}H chemicals shifts were obtained from NMR measurement for peptide HYNIC conjugate: Met (αH=4.52**,** βH=2.15, 2.01 γCH_2=2.64, 2.64 εCH_3=2.13), Leu (αH=4.38**,** βH=1.65, 1.65 γCH_2=1.64, δCH_3=0.94, 0.90), His (αH=4.63**,** βH=3.26, 3.20, γCH_2=2.64, 2.64, 2H=8.12, 4H=7.14), Gly (αH=3.97), Val (αH=4.18**,** βH=2.13, γCH_2=0.97,0.94), Ala (αH=4.35, βH=1.39), Trp (αH=4.70, βH=3.32, 3.19, 2H=7.24, 4H=7.65, 5H=7.17, 6H=7.24, 7H=7.5), Gln (αH =4.37**,** βH=2.13, 2.01, γCH_2=2.38, 2.38), Tyr (αH=4.60**,** βH=3.13, 2.92, 2, 6 H=7.15, 3, 5H=6.86).

The mass analysis of the synthetic molecule indicated that the main peak was related to HYNIC-peptide (m/z =1237.6) which shows half peak in adduction with sodium (m/z = 639.8, $[M + K]^{++}$).

The [^{99m}Tc-HYNIC^{0}, D-Tyr^{6}, D-Trp^{8}] Bombesin [6-14] conjugate with co-ligand including tricine/EDDA was obtained in high radiochemical yield (98.3%) that was stable up to 24 h.. The crude HPLC Chromatographic profile for the ^{99m}Tc/tricine/EDDA-HYNIC-BN conjugate produced with retention time 15.34 min is shown in figure 3.

After 24 h incubation in human serum the radiochemical purity remained >90% (Fig. 4). In internalization study, specific uptake of radioligand in PC-3 cell after 1 h was 2.2% ± 0.5% which was increased to 10.9% ± 1.3% after 4 h. As it shows the significant differences of uptake between blocked and unblocked cells in various time periods are very noticeable ($p < 0.05$) (Fig. 5).

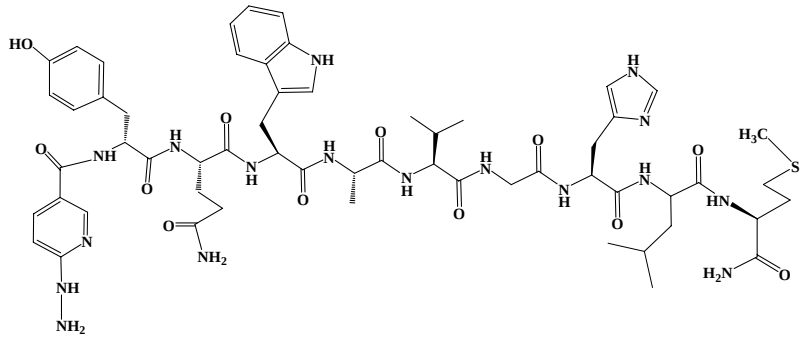

Figure 1. Representative Structure of [HYNIC^0, D-Tyr^6, D-Trp^8] - Bombesin [6-14] NH_2

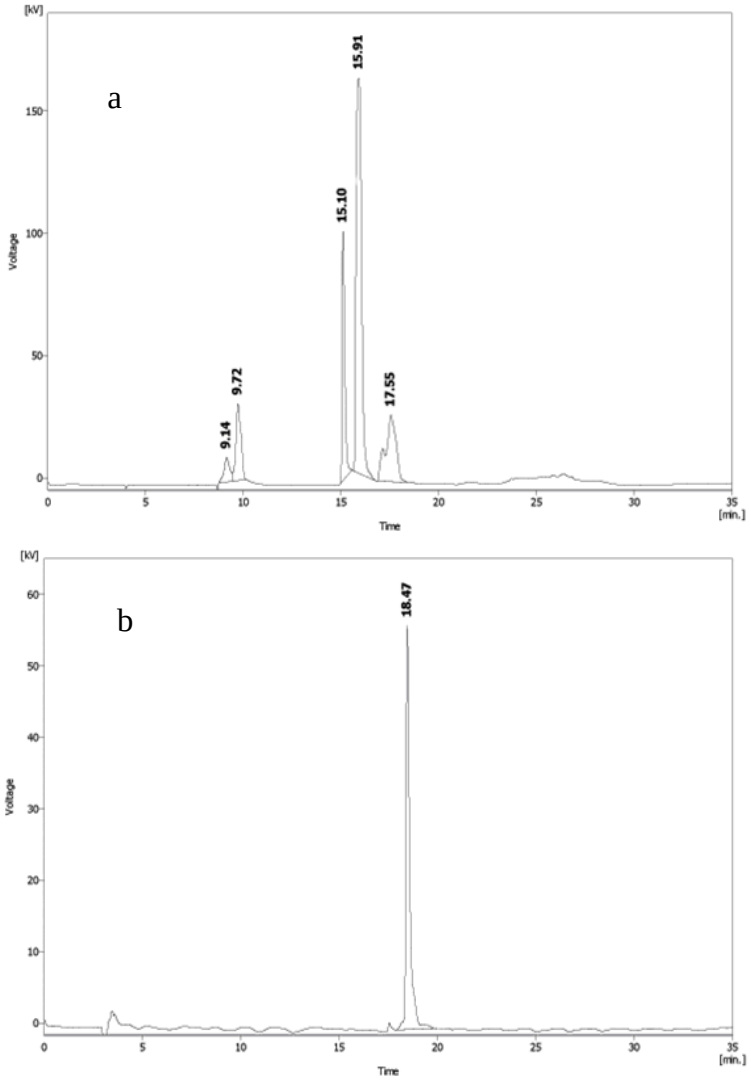

Figure 2. RP-HPLC profile of the [HYNIC^0, D-Tyr^6, D-Trp^8] Bombesin [6-14] NH_2 before (a) and after (b) purification

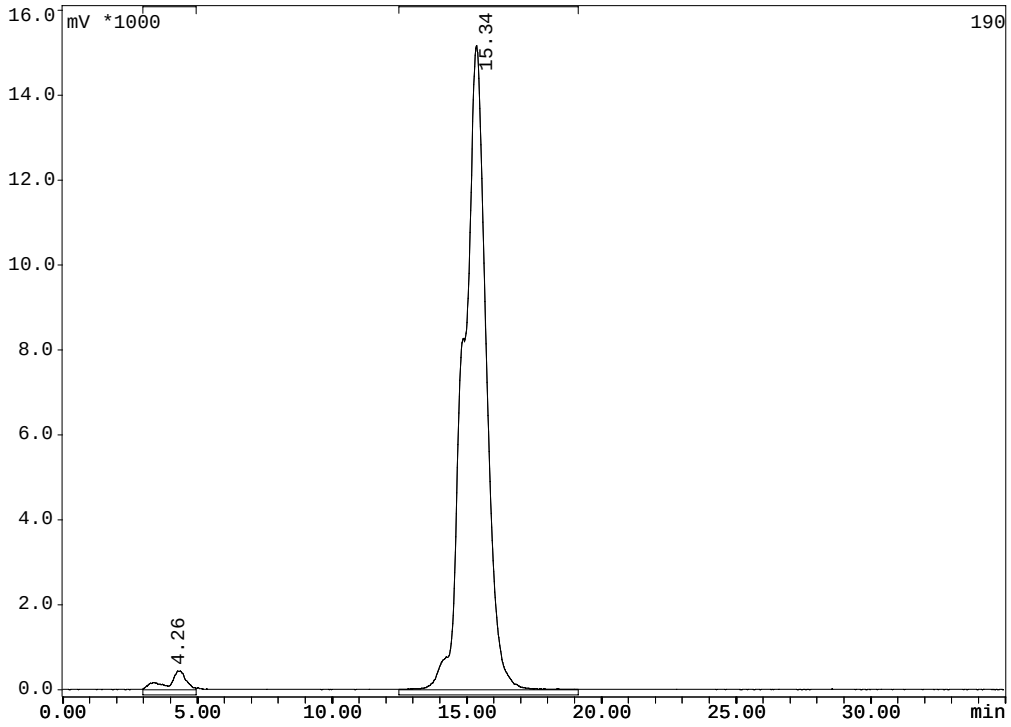

Figure 3. RP-HPLC profile of [^{99m}Tc/tricine/EDDA-HYNIC^{0}, D-Tyr^{6}, D-Trp^{8}] Bombesin [6-14] NH_2

The results of biodistribution in rats are summarized in Table 1. The tissue distribution of radioactivity at 4 h after injection exhibited a rapid clearance from the blood and most tissues predominantly by renal excretion. The highest non-specific uptake was found in kidneys. A significant uptake of radioactivity was observed in the pancreas which expresses GRP receptors. The specificity was confirmed by the receptor blocking study in which the previous injection of cold peptide diminished the uptake of activity in pancreas. Reduction uptake percentage was 65% in the pancreas (0.37% ID/g vs. 0.13% ID/g, $p < 0.05$). The uptake in non-targeted tissues was not significantly reduced by the blocking dose.

We are working to find a new conjugate to be labeled in high yield and in a very short time. It is necessary that the metallic radionuclide be stable under in vivo conditions especially in serum.

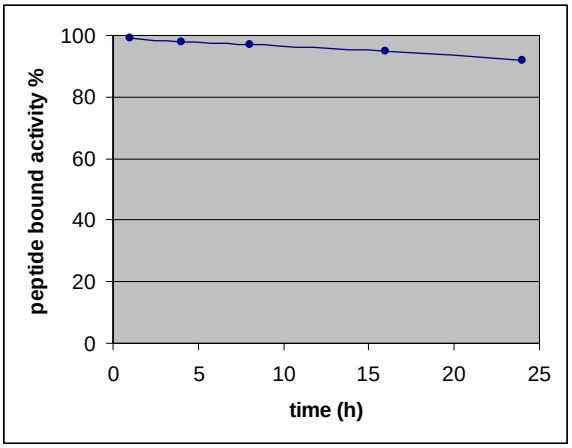

Figure 4. Stability of [^{99m}Tc/tricine/EDDA-HYNIC^{0}, D-Tyr^{6}, D-Trp^{8}] Bombesin [6-14] NH_2 in human serum up to 24 h.

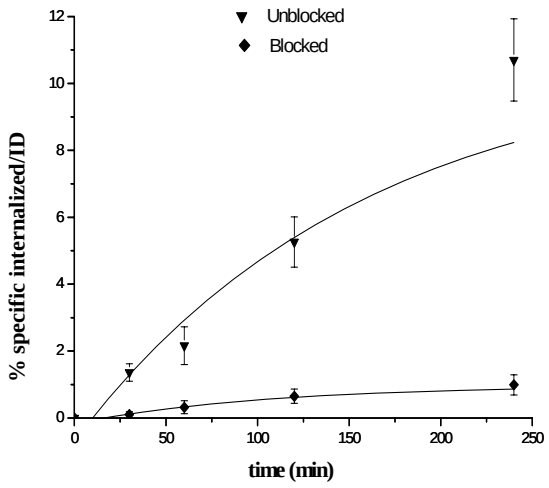

Figure 5. Internalization rate of [^{99m}Tc/tricine/EDDA-HYNIC^{0}, D-Tyr^{6}, D-Trp^{8}] Bombesin [6-14] NH_2 into unblocked and blocked PC-3 cells.

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 (23, 24). The use of coligand allows easy modification of the hydrophilicity and pharmacokinetics of ^{99m}Tc-labeled peptide conjugates. Modified bombesin analogue was prepared and it was easily labeled and its labeling yield was high. The stability of radiopeptide in human serum up to 24 h after its labeling could be attributed to D-Trp substitution instead of L-Trp which in turn may enhance metabolic stability.

Efficient internalization is an important factor for optimization of a radiopharmaceutical in targeted diagnostic and radiotherapy in nuclear medicine. The rapidly, easily and specifically internalized radiopeptide in PC-3 cells indicate that D-Tyr^{6} can be a good spacer to reduce the influence of HYNIC moiety in receptor binding affinity and internalization of peptide. Labeling of the peptide with bifunctional chelating agent HYNIC and tricine/EDDA coligands modifies the lipophilic and pharmacokinetic properties of peptide, resulted a bombesin analogue with low hepatobiliary clearance and predominantly renal excretion. This is an important result as compared to the most of the ^{99m}Tc-labeled bombesin analogues which have a tendency to accumulate in the liver and intestine because of their high lipophilicity (25, 26). Another important advantage of this work is feasibility to prepare a freeze dried kit formulation for routine clinical use in nuclear medicine.

Table 1. Biodistribution in rats (% injected dose per gram organ ± SD, n = 3), *bl* = blocked

Organ	1hrs	4hrs	4hrs bl	24hrs
Blood	0.27±0.05	0.11±0.08	0.12±0.08	0.09±0.04
Bone	0.13±0.02	0.07±0.02	0.09±0.03	0.05±0.02
Kidneys	5.84±1.09	4.96±1.26	5.09±1.11	3.86±0.89
Adrenals	0.58±0.02	0.11±0.08	0.13±0.03	0.95±0.21
Pancrea	1.14± 0.11	0.37±0.08	0.13±0.05	0.08 ±0.04
Spleen	0.16±0.03	0.14±0.05	0.12± 0.04	0.82±0.07
Stomach	0.24± 0.16	0.09±0.03	0.1±0.04	0.31±0.08
Intestines	4.49± 0.46	0.96±0.15	0.88±0.07	0.98±0.03
Liver	0.17± 0.08	0.11±0.03	0.13±0.03	0.16±0.02
Lung	0.27±0.05	0.14±0.02	0.13±0.04	0.28±0.08
Heart	0.20±0.01	0.11±0.02	0.12±0.03	0.09±0.03
Muscle	0.08±0.02	0.05±0.01	0.16±0.06	0.02 ±0.01

CONCLUSION

In this study, labeling of a novel conjugate with ^{99m}Tc was performed using coligand. Reaction time was very short and facile, making this an ideal radiopharmaceutical for clinical studies in nuclear medicine. Furthermore, this conjugate prepared by tricine/EDDA exchange labeling demonstrated excellent radiochemical stability even up to 24 hours post incubation. The prepared ^{99m}Tc-HYNIC-BBN conjugate has promising characteristics for the diagnosis of malignant tumors.

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