New Freeze-dried Kit for Diagnosis of Bombesin Receptor Expressing Tumors

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ABSTRACT

Introduction: It has been shown that some primary human tumors and their metastases, including prostate and breast tumors, over-express gastrin-releasing peptide (GRP) receptors. Bombesin is a neuropeptide with a high affinity for these GRP receptors. The purpose of this study was to prepare and evaluate the characteristics of a new Freeze-dried kit, [6-hydrazinopyridine-3-carboxylic acid (HYNIC)]-GABA-Bombesin [7-14] NH_2 designed for the labeling with ^{99m}Tc using tricine and EDDA 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. The internalization rate was studied in GRP receptor expressing PC-3 cells. Biodistribution of radiopeptide was studied in rats.

Results: Radiolabeling was performed at high specific activities, and radiochemical purity was >98%. The stability of radiolabeled peptide in human serum was excellent. *In vitro* studies showed >14% of activity was specific internalized into PC-3 cells up to 4 h. After injection into rat biodistribution data showed a rapid blood clearance, with renal excretion and specific binding towards GRP receptor-positive tissues such as pancreas (1.15±0.19% ID/g after 4 h).

Conclusion: [^{99m}Tc-HYNIC]-GABA-Bombesin [7-14] NH_2 showed favorable radiochemical and biological characteristics which make our new designed labeled peptide conjugate as a very suitable agent for diagnostic purposes in malignant tumors.

Keywords: Bombesin; HYNIC; Freeze-dried Kit; GRP receptors; Tumor

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INTRODUCTION

The molecular basis for the use of radiopeptides was found to be that peptide receptors are over-expressed by certain tumors (1, 2). Several peptide receptors such as somatostatin, neurotensin and bombesin receptors have attracted considerable interest in recent years. Bombesin is a 14-aminoacid peptide isolated from frog skin. The mammalian counterparts of the frog peptide are neuromedin B (NMB) and gastrin-releasing peptide (GRP). Over-expression of receptors for both NMB and GRP have been reported to be found on the cell surfaces of several malignant tissues (3, 4), particularly in the cases of lung cancer (5), colon cancer (6), prostate cancer and breast cancer (7-10). Up to now, many types of radiolabeled BBN analogues have been designed to target GRP receptor expressing tumors. For example, ^{99m}Tc, ^{111}In and ^{67}Ga labeled BBN analogues have been developed for SPECT and ^{64}Cu and ^{68}Ga labeled BBN analogues for positron emission tomography (PET) imaging (11-17). ^{90}Y and ^{177}Lu labeled analogues have been described as promising tools for targeted radiotherapy of these tumors (18). Some radiolabeled bombesin analogs have been studied on metastatic breast and prostate cancer. ^{99m}Tc-bombesin correctly diagnosed 12 of 15 breast cancers, while scintigraphy was negative in three of benign lesions (19). Using ^{99m}Tc-BBN, Scopinaro et al diagnosed eight of primary prostate cancers and correctly detected node invasion in three patients, in whom computed tomography and magnetic resonance imaging were negative or inconclusive. Moreover, they demonstrated two of true-negative scans in benign prostate lesions (13).

Whereas ^{99m}Tc is still most ideal for diagnosis, based on its favorable nuclear properties, a wide variety of chelating agents like N_3S (^{99m}Tc-RP527), N_2S_2, N_4 (^{99m}Tc-Demobesin 1), P_2S_2, HYNIC and carbonyl have been used for development of ^{99m}Tc-BBN analogues (20-23). One of the bifunctional chelating ligand that has received considerable interest is HYNIC. The use of the ^{99m}Tc-HYNIC core was first reported for the labeling of IgG and since then, has been conjugated to various biomolecules including antibodies and peptides (24). Since HYNIC could only occupy one or two coordination position on the radionuclide, coligands are necessary to complete the coordination sphere of the technetium core (25). Usually a co-ligand, such as tricine or ethylenediamine diacetic acid (EDDA), is included in the ^{99m}Tc labeling of HYNIC conjugates. The co-ligand can sometimes play an important role in the stability, lipophilicity, clearance property and protein binding potency of the radiolabels. For example, while providing higher labeling efficiency, the tricine coligand also provides higher non-specific protein binding potential over the EDDA complexes.

It has been shown that 7-14 amino acid sequence of BBN with amidated C-terminus is necessary for receptor binding affinity and N-terminal of peptide can be used for labeling (26). As we know in development of new BBN analogues ^{99m}Tc-chelator complex could coupled directly or via a spacer group to the N-terminus of the peptide. It has been shown the best results achieved by conjugates which ^{99m}Tc complex placed at a certain distance from the N-terminus of peptide (27). Therefore, most radiolabeled BBN analogues are based on 7–14 amino acid sequence, coupled with a chelator through a spacer group at the N-terminus of the peptide (28, 29). Also we recently reported the evaluation of a new somatostatin analogues labeled via bifunctional chelating agents HYNIC and EDDA/tricine as coligands (30). In continuation of our efforts to create a new ^{99m}Tc-labelled peptide for tumor targeting we have recently developed and evaluated the radiolabeled peptide [^{99m}Tc/HYNIC^{0}, D-Tyr^{6}, D-Trp^{8}] bombesin (6-14) NH_2 which

internalized rapidly into GRP receptor-positive tumor cells (31, 32). To extend our previous study and to increase the stability and hydrophilicity, and in order to improve the tumor uptake of the labeled peptide in tumor bearing mice we prepared a EDDA/tricine complex of ^{99m}Tc-HYNIC-Bombesin [7-14] peptide which gamma amino butyric acid (GABA) as a three carbon chain spacer between HYNIC and N-terminus of the peptide.

Here we present data on the synthesis of HYNIC-GABA-Bombesin [7-14] NH_2 and preparation of a freeze-dried kit formulation which could be labeled with ^{99m}Tc using tricine and EDDA as coligands. In addition we studied stability in human serum, receptor bound internalization in PC-3 cells and in vivo tissue uptake and biodistribution of this freeze-dried kit.

METHODS

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 (24). Other reagents were purchased from Fluka, and used without further purification.

The reactive side chains of the amino acids were masked with one of the following groups: Trp, t-butoxycarbonyl (Boc); His and Gln, Triphenylmethyl (Trt). The cell culture medium was Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), amino acids, vitamins and penicillin/streptomycin from Gibco. 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 multiwavelength detector and a flow-through Raytest-Gabi γ-detector. CC 250/4.6 Nucleosil 120-5 C18 column from Teknokroma was used for analytical HPLC, and a VP 250/10 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 analytical HPLC, Gradient I was used: 0 min 95% A (5% B), 5 min 95% A (5% B), 25 min 0% A (100% B), 27 min 0% A (100% B), 30 min 95% A (5% B), flow = 1 mL/min, γ = 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), 25 min 80%A (20% B), flow = 2 mL/min, γ = 280 nm. Mass spectrum was recorded on an Agilent 1100/ Bruker Daltonic (Ion trap) VL instrument (LC/MS). Quantitative gamma counting was performed on an ORTEC Model 4001 M γ-system well counter.

Synthesis

The peptide was synthesized by standard Fmoc solid phase synthesis on Rink Amide MBHA resin with substitution, 0.69 mmol/g. 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 diisopropylamine (DIPEA) in Dimethylformamide (DMF). Completeness of coupling reactions was monitored by the Kaiser test and the Fmoc groups were removed by adding 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). The peptide HYNIC conjugate was removed from the resin and amino acid side chains were also deprotected by treatment with a cocktail of trifluoroaceticacid (TFA), triisopropylsilane

and water (95:2.5:2.5). After removing the organic solvents in vacuum, the crude product was precipitated with cold Petroleum ether and Diisopropyl ether (50:50). The crude peptide HYNIC conjugate was dissolved in water/methanol and purified by semi-preparative RP-HPLC; then the purified product was characterized by LC/MS and analytical HPLC.

Kit formulation

One milliliter of a solution containing 5 mg EDDA, 15 mg tricine, 40 µg $SnCl_2$ (20 µl of 2 mg/ml $SnCl_2$, $2H_2O$ in nitrogen-purged 0.1 M HCl) and 20 µg purified HYNIC-peptide conjugate with a final pH of 7 was filtrated into a glass vial and freeze dried.

Labeling and quality control

Radiolabeling of the kit was performed by adding 0.5 ml 0.9% saline in an evacuated vial, and the mixture was allowed to preincubate for 5 min. Then, 1 GBq $^{99m}TcO_4^-$ in 0.5 ml saline was added to the vial and incubated for 10 min at 100°C. After cooling at room temperature, the labeled peptide was analyzed by analytical HPLC (Gradient I) and ITLC on silica gel 60 (Merck) using different mobile phases: 2-butanone for free $^{99m}TcO_4^-$ (Rf=1), 0.1 M sodium citrate (pH=5) to determine the non-peptidebound ^{99m}Tc coligand with $^{99m}TcO_4^-$ (Rf=1) and methanol/1M ammonium acetate 1/1 for ^{99m}Tc colloid (Rf=0).

Stability in human serum

The stability and the affinity of the labeled kit 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.

Cell culture

The PC-3 cells (obtained from Pasteur Institute of Iran) were cultured in DMEM supplemented with 10% FBS, 2 mM glutamine and penicillin–streptomycin. Cells were maintained in a humidified 5% CO_2/air atmosphere at 37°C. For all cell experiments, the cells were seeded at a density of 1 million cells per well in 6-well plates and incubated overnight with internalization medium (DMEM with 1% FBS).

Internalization

Medium was removed from the 6-well plates, and cells were washed once with 2 ml of internalization medium (DMEM 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) of labeled kit 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/L bombesin. The cellular uptake was stopped at appropriate time periods (30 min, 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).

Biodistribution

Animal experiments were performed in compliance with the regulations of our institution and with generally accepted guidelines governing such work. An activity of 20 MBq (0.35 nmol) of ^{99m}Tc-HYNIC-GABA-Bombesin [7-14] NH_2 was injected *via* the femoral vein. In order to determine the non-specific uptake of the radiopeptides, in receptor-positive organs, a group of three

animals were injected with 100 µg cold peptide in 50 µl saline as a co-injection with the radiopeptides (blocked animals). After 1, 4 and 24 h, the rats in groups of three animals were killed, organs of interest were collected, weighed and radioactivity was measured in a gamma-counter. Also the 1 h post injection image was obtained at a small area mobile gamma camera (Siemens).

Statistical methods

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

HYNIC-GABA-Bombesin [7-14] NH_2 was synthesized by Fmoc strategy supplying an overall yield of 45% based on the removal of the first Fmoc group after cleavage, purification and lyophilization (Figure 1). The purity was 97.3% as confirmed by HPLC method as a single peak in retention time of 18.12 min (Figure 2). The mass analysis of the synthetic molecule indicated that the main peak was related to HYNIC-peptide (m/z =1159.57) which shows half peak in adduction with potassium (m/z = 600.3, $[M + K]^{++}$).

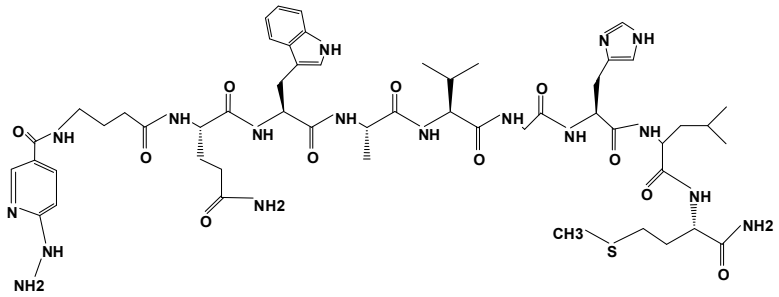

Figure 1. Structural formulae of HYNIC-GABA-bombesin [7-14] NH_2.

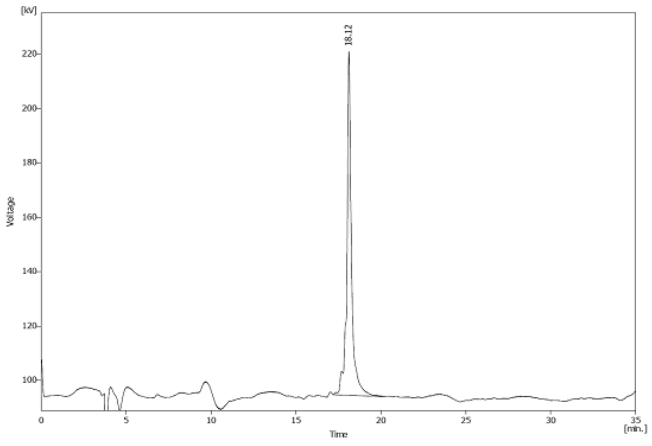

Figure 2. RP-HPLC profile for HYNIC-GABA-Bombesin [6-14] NH_2 after purification.

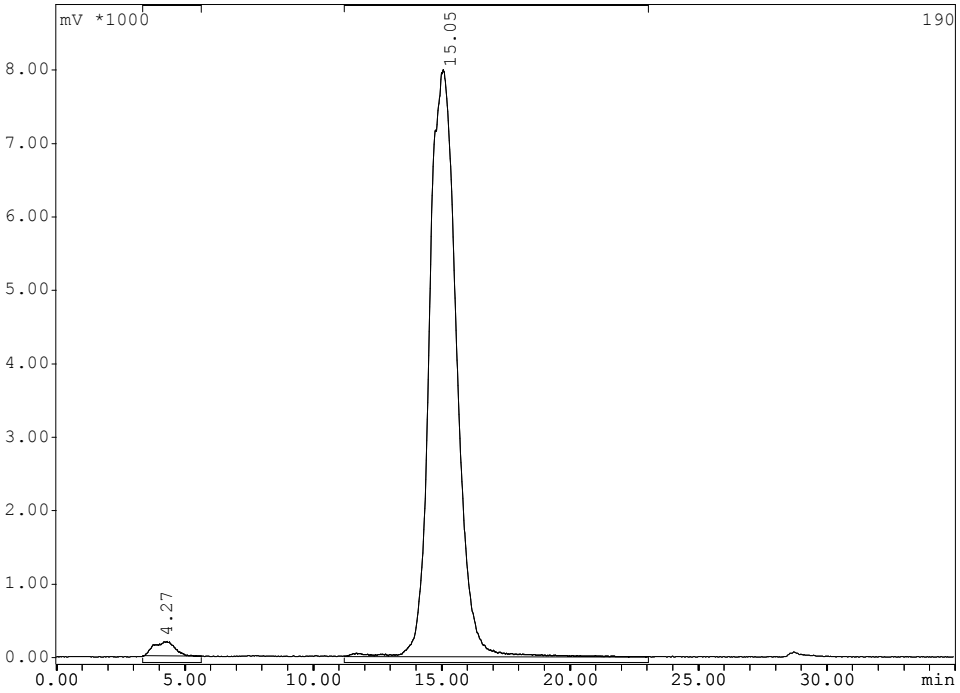

Figure 3. RP-HPLC analysis of ^{99m}Tc-HYNIC-GABA-Bombesin [7-14] NH_2, elution times are 4.27 min for free technetium and 15.05 for labeled peptide.

The labeling yield of ^{99m}Tc-HYNIC-GABA-Bombesin [7-14] NH_2 prepared from lyophilized kit was >98%, acquired via HPLC and also ITLC at a specific activity of 80 GBq/ µmol. The HPLC elution times (Gradient I) were 4.27 min for $^{99m}TcO_4^-$ and 15.05 min for ^{99m}Tc-peptide (Figure 3).

After 24 h incubation in human serum the radiochemical purity remained >90%. Figure 4 shows the result in respect of the time-dependent and specific internalization of the radioligand into PC-3 cells. During 60 min, the radioligand showed 4.98±0.85% specific cell uptake, which increased to 14.13±0.61% up to 4 h. In all experiments, the internalization was strongly reduced in the presence of excess cold. In fact, nonspecific internalization was 0.56±0.31% after 4 h, and the surface-bound peptide (acid removable) was 1.2± 0.44% of the added activity after 4 h.

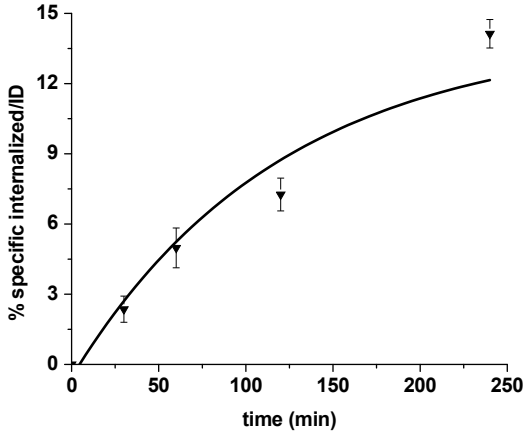

Figure 4. Internalization rate of ^{99m}Tc-HYNIC-GABA-Bombesin [7-14] NH_2 into PC-3 cells.

Results from biodistribution studies using the ^{99m}Tc-labeled peptide are presented in Table 1 as the percentage of injected dose per gram of tissue (% ID/g). ^{99m}Tc-HYNIC-GABA-Bombesin [7-14] NH_2 displayed rapid blood clearance with 0.10±0.02% ID/g at 4 hour. Fast clearance from the gastrin-releasing peptide receptor-negative tissues except the kidneys was found as well. Labeled peptide shows high uptake values in the gastrin-releasing peptide receptor-positive organs. By blocking the receptor through prior injection of cold peptide, the uptake in pancreas is diminished and this confirms the specificity of radioconjugate.

Uptake reduction was 91% (1.19% ID/g vs. 0.11% ID/g at 4 h). On the other hand, the uptake reduction in non-targeted tissues due to blocking dose was not significant. Typical Scintigram of the normal rats 1 h post injection also shows that kidneys are the main excretory organs for labeled peptide (Figure 5).

Table 1. Biodistribution in mice (% injected dose per gram organ ± SD, n = 3)

Organ	1 h	4 h	4 h block	24 h
Blood	0.20±0.09	0.10±0.02	0.12±0.02	0.06±0.01
Bone	0.34±0.1	0.25±0.07	0.21±0.07	0.05±0.01
Kidney	3.88±0.48	2.12±0.43	1.97±0.21	0.71±0.12
Adrenal	0.85±0.12	1.11±0.41	0.30±0.09	0.84±0.13
Pancreas	1.51±0.18	1.19±0.14	0.11±0.03	0.38±0.05
Spleen	0.59±0.3	0.48±0.15	0.43±0.12	0.10±0.03
Stomach	0.17±0.03	0.12±0.021	0.08±0.01	0.10±0.02
Intestine	0.43±0.09	0.32±0.06	0.12±0.03	0.06±0.01
Liver	0.31±0.08	0.26±0.02	0.31±0.06	0.10±0.03
Heart	0.23±0.04	0.11±0.03	0.12±0.04	0.04±0.01
Muscle	0.20±0.06	0.07±0.01	0.09±0.01	0.03±0.01

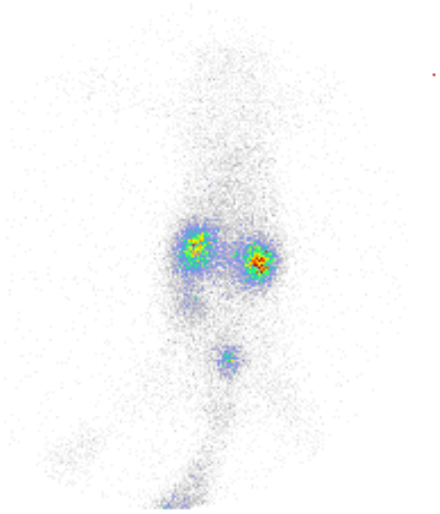

Figure 5. Scintigram of normal mice after 60 min post injection of ^{99m}Tc-HYNIC-GABA-Bombesin [7-14] NH_2.

DISCUSSION

GRP receptors were shown to be over-expressed on a variety of human tumors such as breast and prostate cancers. As we previously reported bombesin analogue [^{99m}Tc-HYNIC^0, D-Tyr^6, D-Trp^8] Bombesin (6-14) NH_2 is capable of visualizing GRP receptor-positive tumors *in-vivo* (31, 32). In the present study, we evaluated a new Freeze-dried kit which contains a bombesin [7-14] peptide sequences. In order to improve binding affinity, we attached HYNIC chelator *via* a GABA spacer to the N-terminus of the peptide.

A variety of BFCA have been used in the labeling of proteins and peptides with ^{99m}Tc (20), and among those, HYNIC is the best alternative because it can achieve labeling in high specific activity followed by using various coligands, which permit control of the hydrophilicity and pharmacokinetics of the labeled peptide (25, 33). In the group of different coligands, tricine gives the best radiolabeling efficiency but the complex is not stable, particularly in dilute solutions (34). 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 (35, 36).

In this study we used a Freeze-dried kit with 20 μg of HYNIC-peptide followed by using tricine and EDDA together as coligand in amounts of 15 mg and 5 mg in final volume of labeled solution respectively. We obtained high radiochemical yield >98% with very low amount of ^{99m}Tc-pertechnetate (<0.2) ^{99m}Tc-radiocolloid (<0.5) and ^{99m}Tc-coligands (<1.0). In comparison to those reports regarding ^{99m}Tc-tricine-HYNIC complex instability (25), our new labeled kit was stable up to 24 h post labeling period at room temperature. The high labeling yield and the stability may be attributed to optimization of kit preparation and our labeling method.

High rate of internalization was observed for our labeled kit (14.13±0.61% up to 4 h) which was not unexpected since Bombesin [7-14] NH_2 sequence offers agonistic property to the compound. Previous studies of ^{111}In-DOTA-8-Aoc-BBN [7-14] NH_2 (18) and [^{111}In]-DTPA or [^{111}In]-DOTA-GABA-[D, Tyr^6, β-Ala11, Thi13, Nle14] BN (6-14) [BZH1 and BZH2] (16) also demonstrated internalization and receptor mediated trapping of labeled compounds (18). In comparison to our pervious works this new labeled kit showed increased rate of internalization as compared with [^{99m}Tc/tricine/HYNIC^0, D-Tyr^6, D-Trp^8] BN (6-14) NH_2 (14.13 ± 0.61% versus 10.7 ± 1.2% after 4 h) (31, 32). It could be due to replacement of a three carbon chain spacer (GABA) instead of D-Tyr^6. By placing bifunctional chelating agent farther from the receptor binding region, the negative effect of chelator could be reduced.

New labeled kit showed fast blood clearance with negligible amount remaining in the

blood after 4 hours (<0.13 % ID/g). Clearance from GRP receptor negative tissues was also rapid except from the kidneys. In comparison with our pervious study (31, 32), due to improvement in binding affinity of our new labeled kit, increased activity in pancreas was observed (1.51±0.18 %ID/g versus 1.04±0.11 %ID/g at 1 h). The uptake in pancreas, adrenal, stomach and intestine was specific and receptor mediated, as shown by the co-injection of cold peptide, indicating that these organs are also GRP receptor positive.

As most of the ^{99m}Tc-labeled bombesin analogues have a tendency to accumulate in the liver and intestine with a low kidney excretion due to their high lipophilicity (37), our new labeled kit demonstrates a good improvement in renal excretion. Another important advantage of this work is the preparation of new freeze dried kit formulation that makes it ideal for routine clinical use in nuclear medicine.

CONCLUSION

In this study, we have shown preparation of a new freeze-dried kit for diagnosis of GRP receptor-positive tumors. Labeling of kit with ^{99m}Tc was performed by using coligand in high specific activity. Labeled kit showed high accumulation in pancreas as a positive GRP receptors targeted tissue followed by excretion *via* the kidney. These promising characteristics make our new designed kit formulation as a very suitable agent for diagnosis of GRP receptor-positive tumors.

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