

IN VITRO AND IN VIVO STUDIES IN *Balb-c* AND *Nude* MICE OF A NEW ¹⁷⁷LU-BOMBESIN ANALOG DEVELOPED FOR PROSTATE TUMOR DIAGNOSIS AND TREATMENT

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ABSTRACT

In this work we describe the radiolabeling with ¹⁷⁷Lu and some properties of the novel bombesin analog BBNp6 – DOTA-X-BBN(6-14), where X is a spacer of six aminoacids. Bombesin (BBN) is an analog of human gastrin releasing peptide (GRP) isolated from the skin of the frog *Bombina bombina* in 1970. Development of radiolabeled BBN derivatives as agents for diagnostic imaging and systemic radiotherapy has increased considerable because of the observation that GRP receptors (GRPr) are over-expressed in a variety of human tumor cells, such as prostate tumor cells. ¹⁷⁷Lu-labeled peptides are attractive due to the excellent radiophysical properties and commercial availability of the radiometal. BBNp6 was labeled with high yield after reacting with 92.5 MBq of ¹⁷⁷LuCl₃ at 90 °C for 30 minutes and this mixture kept stable for more than 96 hours at 4 °C and 1 hour in human plasma. *In vivo* studies showed a multicompartmental distribution model with fast blood clearance, mainly performed by renal pathway. In addition, ¹⁷⁷Lu-BBNp6 showed high affinity for PC-3 tumor xenografts, but not for pancreas and intestine (GRP positive tissues), suggesting its specificity and usefulness for prostate tumor treatment. Moreover, scintigraphic images showed that this derivative can also be a tool in this tumor diagnosis. So, BBNp6 is a promising radiopharmaceutical for prostate tumor imaging and treatment.

1. INTRODUCTION

Radionuclides coupled to receptor-specific peptides are currently under investigation in clinical trials involving different tumors. They specifically localize receptors overexpressed on the plasma membrane and then internalize into cells. The prototypes of these peptides are somatostatin analogs [1] and the success in the area of somatostatin receptor-positive tumor targeting with diagnostic and therapeutic radionuclides has stimulated research toward radionuclide targeting of alternative receptor systems overexpressed in tumors [2].

In designing radiometal-based radiopeptides for cancer diagnosis and treatment, important factors to consider are half-life, mode of decay, cost and availability of the radioisotope and

the chemical and biological properties of the labeled molecule. In the field of the radioisotope, the application of ^{177}Lu isotope in medicine is spreading in a last few years. This radiolanthanide has been finding a continuously wider use in diagnosis and therapy. This increasing is probably due to its good radiation properties. Its half-life of 6.65 days permits to apply sophisticated procedures to synthesize and purify the radiopharmaceuticals requiring the use of more time and work. Besides, its beta radiation of 498 keV maximum energy is very suitable for cancer therapy ensuring the interacting range in a human tissue of about 670 μm . The presence of modestly energetic and abundant (11%) gamma-radiation of 208 keV makes it suitable for gamma scintigraphy. As with other radiolanthanides, it is relatively easy to conjugate the ^{177}Lu numerous biologically active compounds [3].

In the field of radiolabeled molecules, a high number of regulatory peptides receptors were shown to be overexpressed in various human tumors. They are promising targets for molecular imaging and targeted therapy of cancer, because they are located on the plasma membrane and, upon binding of a ligand, the receptor-ligand is internalized. Among these most relevant peptide receptors, the bombesin receptors are of major interest [4]. Bombesin peptide (BBN) is a 14-amino acid analog of human gastrin releasing peptide (GRP) originally isolated from the skin of the frog *Bombina bombina* in 1970. There are known four receptor subtypes of BBN, including the neuromedine B receptor (subtype 1), the GRP receptor (GRPr, subtype 2), the orphan receptor (subtype 3) and the BBN receptor (subtype 4) [5]. GRPr expression is normally restricted to nonneuroendocrine tissues of the pancreas and breast, and neuroendocrine cells of the brain, gastrointestinal tract, lung and prostate, but it is not normally expressed by epithelial cells present in colon, lung or prostate [6]. In addition, gastrin-releasing peptide receptors are predominantly expressed in different human tumors, such as prostate cancer, gastrinoma, and breast cancer. These findings provide a possibility to apply bombesin-like peptides as a vehicle for delivery cytotoxic radiation doses into tumor cells [4].

Studies with bombesin agonists and antagonists radiolabeled with lutetium-177 have been reported and have shown to reduce tumor growth in mice. Despite the variety of compounds synthesized, one with the optimal characteristics for systemic radiotherapy – including maximal tumor uptake and retention and minimal nontumor tissue uptake and retention – has yet to be reported. Most of the studied analogs exhibit high abdominal accumulation, specially in pancreas and intestine [6-10]. This abdominal accumulation may represent a problem in clinical use of radiolabeled bombesin analogs probably due to serious side effects to patients. The goal of the present work as to radiolabel with ^{177}Lu and to describe some properties of the novel bombesin analog BBNp6 – DOTA-X-BBN(6-14), where X is a spacer of six aminoacids.

2. MATERIALS AND METHODS

DOTA-X-BBN(6-14) was provided from piChem and $^{177}\text{LuCl}_3$ was obtained from IBD (Netherlands). All other chemicals and reagents required for experiments were of analytical grade and were purchased from Sigma Aldrich Co. (USA) or Merck Co. (Germany).

2.1. Preparation of radiotracer

Preliminary studies were done to establish the ideal labeling conditions of labeled BBNp6. All reagents were prepared with Chelex 100 treated free metal water. Briefly, BBNp6 (20 µg), 0.4 mol.L⁻¹ sodium acetate buffer pH 4.5 and 92.5 MBq (2.5 mCi) of ¹⁷⁷LuCl₃ (specific activity 871 – 920 GBq/mg) were heated at 90 °C for 30 minutes. The radiochemical purity was determined at the end of the reactions as described forward.

2.2. Radiochemical purity determination

Instant thin layer chromatography (ITLC-SG) was applied to determine free lutetium, with citrate/citric acid buffer pH 5.0 as solvent (retention factor - R_f - of labeled peptide was 0.1-0.3 and R_f of free lutetium was 0.9-1.0). Radiochemical purity was also determined by high liquid performance chromatography (HPLC - Shimatzu) using a reversed phase C₁₈ column (Waters, 4.0 x 150 mm, 5 µm) with radioactivity (Shell) detection, flow rate of 1.5 mL/minute with a linear gradient of 10-90% (v/v) 0.1% TFA / acetonitrile and 0.1% TFA / H₂O for 15 minutes.

2.3. Stability of radiolabeled BBNp6

To determine the ¹⁷⁷Lu-BBNp6 *in vitro* stability the preparation was stored at 4 °C for different times (1 to 7 days) or human serum samples were spiked with ¹⁷⁷Lu-BBNp6 and incubated at 37 °C for 1, 4 and 24 hours, followed by ITLC-SG analysis. All experiments were performed in triplicate.

2.4. *In vivo* studies

2.4.1 Animals

Animal studies were performed in accordance with United Kingdom Biological Council's Guidelines on the Use of Living Animals in Scientific Investigations as well as institutional guidelines. Male *Balb-c* mice (4 to 8 weeks old, 20-25 g weight) and male *Nude* mice (4 to 8 weeks old, 15-20 g weight) used for *in vivo* experiments were provided by the Animal Facility of IPEN.

2.4.2 Biodistribution and pharmacokinetic studies in healthy *Balb-c* mice

The radiopeptide (0.185 MBq/100µL/mouse) was injected intravenously in mice tail vein. After different time intervals (1, 4 and 24 hours p.i.), the animals were sacrificed in groups of five and the blood was collected. Then, the mice were dissected and vital organs were isolated, weighed and their respective radioactivities were measured in an automatic gamma counter (Packard). The biodistribution of labeled BBNp6 was calculated as percentage uptake of injected activity per organ (% IA) and per gram of organ (% IA/g).

Pharmacokinetics studies were performed by measuring ^{177}Lu -BBNp6 in blood. Blood samples were collected 1, 5, 30, 60, 120, 240 and 1440 min after the intravenous injection of labeled peptide and their radioactivities were measured as described early. The pharmacokinetics parameters were determined using Biexp software.

2.4.3. Biodistribution studies in *Nude* mice bearing PC-3 tumor

Human prostate cancer PC-3 cell line was grown in RPMI 1640 medium containing 10% (v/v) fetal bovine serum and 1% penicillin-streptomycin. Cells were kept in humidified air containing 5% CO_2 at 37 °C. The cells were grown to 80% confluency, harvested by trypsinization, centrifuged (5 min, 2500 rpm) and then resuspended in PBS.

To analyze the affinity of ^{177}Lu -BBNp6 bombesin derivatives target to human prostate tumor cells (PC-3) *in vivo*, male *Nude* mice were inoculated subcutaneously with human PC-3 cells (2×10^6) in 0.1 mL phosphate-buffered saline [6]. Biodistribution studies were performed after 3 weeks with tumors averaging 0.3 g as described earlier.

2.4.4. Imaging studies

Imaging studies were performed in normal male *Balb-c* and *Nude* mice bearing PC-3 tumor at 30 minutes, 1 and 4 hours post intravenous administration of labeled peptide (37 MBq/100 μL 0.9% NaCl). The anesthetized mice were placed under a gamma camera low-energy high-resolution collimator (LEHR) (Mediso Imaging System, Hungria) and the images were acquired for 180 seconds using a 256x256x16 matrix size and a window set at 208 keV.

2.5. Statistical analysis

Statistical analysis were performed using PRISM 5.0 software (USA). Results were subjected to Student's *t*-test and expressed as mean \pm standard derivation (SD).

3. RESULTS

BBNp6 was radiolabeled with ^{177}Lu with high yield ($98.70 \pm 0.15\%$) and a specific activity of ~ 7.30 MBq/ μg was achieved. The Fig. 1 shows a typical radioactive HPLC profile of ^{177}Lu -labeled BBNp6. The labeled peptide (Retention time - $R_t = 6.55$ minutes) could be clearly separated from free lutetium ($R_t = 1.15$ minutes) and no contamination by $^{177}\text{LuCl}_3$ was observed in the radiolabeling mixture.

A second peak of shorter retention time than the mainly radioactive specie was observed in the HPLC profile of labeled peptide, which probably represent the labeled bombesin derivative resulted from oxidation of the methionine residue and represent less than 10% of total radioactivity present in radiolabeling mixture.

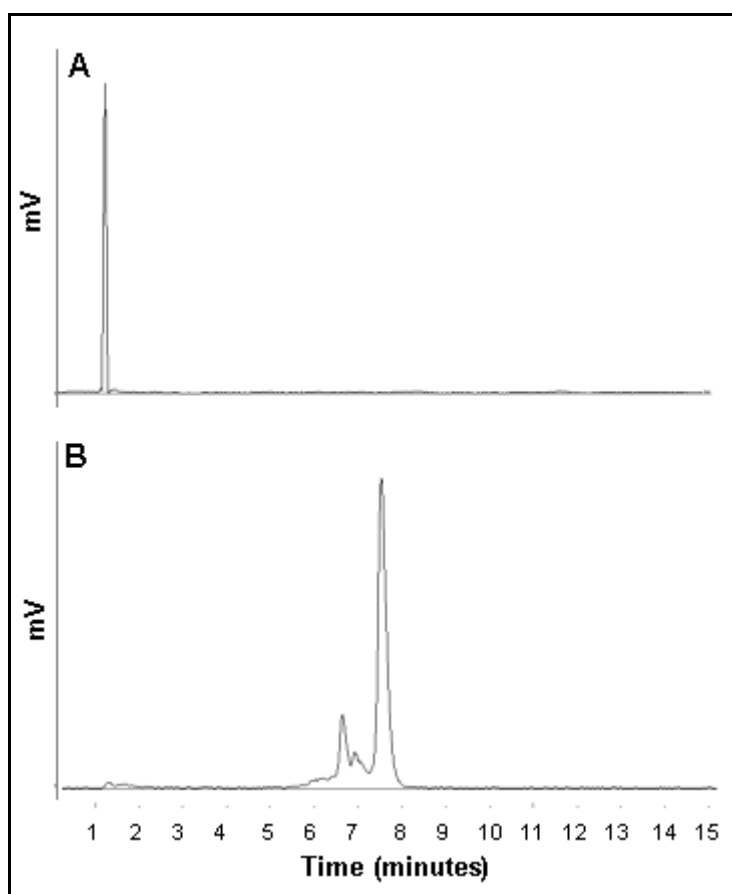


Figure 1. HPLC profile (radioactive) of (A) lutetium-177 and (B) ^{177}Lu -BBNp6.

The stability of labeled peptide was evaluated by instant thin layer chromatography after storage at 4 °C and incubation at 37 °C in human plasma. The Table 1 shows the results obtained from the samples stored at 4 °C for different times. ^{177}Lu -BBNp6 remained stable at this temperature and the radiochemical purity was higher than 90% for more than 168 hours (7 days) of storage.

Table 1. *In vitro* stability of radiolabeled BBNp6 after storing a 4° C for different times.

	Incubation time at 4° C					
	Immediately	24 hours	48 hours	72 hours	96 hours	168 hours
Radiochemical Purity (%)	98.9 ± 0.5	98.1 ± 0.8	96.2 ± 0.9	95.6 ± 0.6	94.1 ± 1.8	91.4 ± 1.1

After incubation of ^{177}Lu -BBNp6 in human fresh serum, differences in the ITLC-SG chromatogram were detected in all time intervals analyzed, suggesting a metabolic degradation of the labeled peptide by serum enzymes. These differences were used to

calculate the radiochemical purity of the preparation in all times analysed, which can be seen in the curve of Fig. 2.

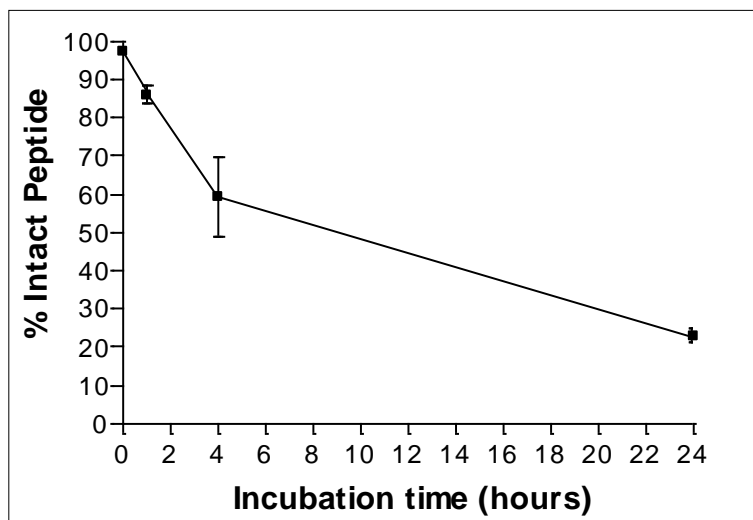


Figure 2. Time-course degradation of ^{177}Lu -BBNp6 by human serum enzymes at 37 °C. The radiochemical purity decreased to 86.1 ± 2.4 , 59.3 ± 10.5 and 23.0 ± 2.0 after 1, 4 and 24 hours of incubation, respectively.

Results from biodistribution studies using the ^{177}Lu -labeled peptide performed with *Balb-c* mice are presented in Fig. 3 as the percentage of injected activity per gram of organ (% IA/g). Appreciable radioactivity could be detected in the kidneys until 24 hours post injection, indicating peptide excretion mainly by renal pathway. Kidneys may be the critical organs for dosimetry. In addition, it could be observed low abdominal accumulation of ^{177}Lu -BBNp6, especially in pancreas.

Bone uptake is commonly assumed as a control of lutetium-labeled compounds stability in *in vivo* assays. This tissue actively uptakes free lutetium-177, being a good indicator of radiochemical purity, mainly at the initial time. Bone uptake of ^{177}Lu -BBNp6 was negligible when compared to pure $^{177}\text{LuCl}_3$ [11], confirming no contamination of free lutetium in the preparation.

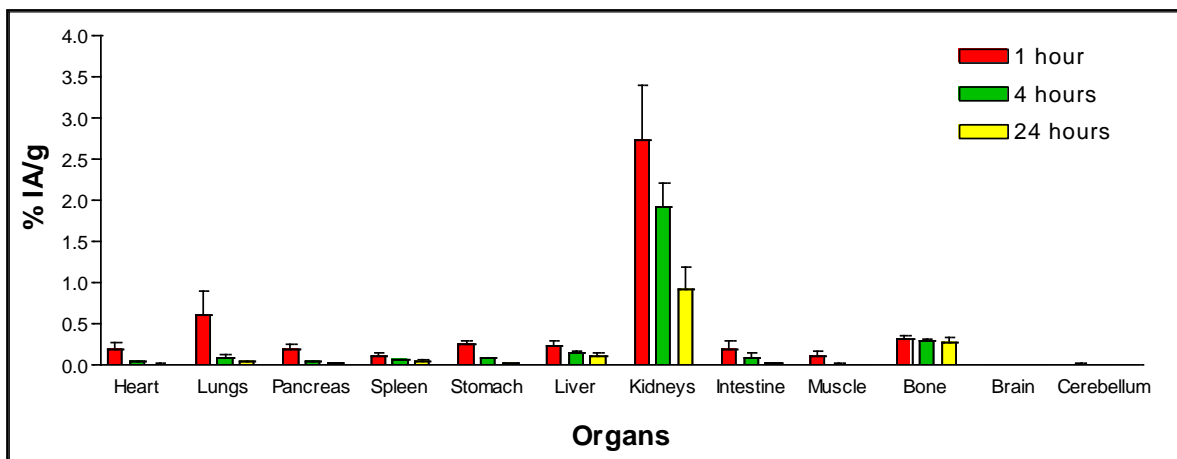


Figure 3. Biodistribution (0.185 MBq) of ^{177}Lu -BBNp6 in healthy *Balb-c* mice (n=5).

To evaluate the pharmacokinetic of ^{177}Lu -BBNp6 in mice we performed kinetics studies by measuring the radiolabeled peptide in blood and the results are expressed in Fig. 4. The amount of ^{177}Lu -BBNp6 present in plasma decreased rapidly and became almost undetectable at 60 minutes post injection. This rapid clearance is performed mainly by renal pathway, as described early in the biodistribution assay.

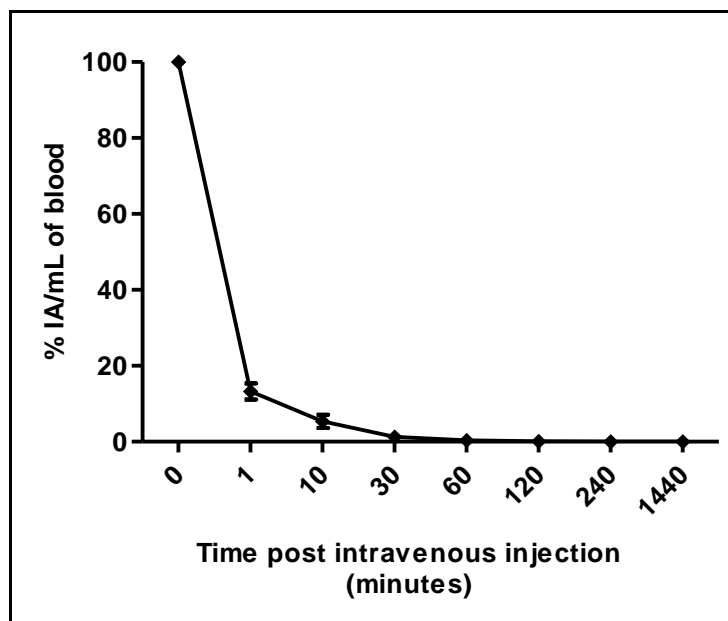


Figure 4. Blood clearance kinetics of labeled BBNp6. Data analysis suggest a multicompartimental biodistribution model.

Although kinetics in blood suggested a multicompartimental biodistribution model, Biexp software was used to calculate the pharmacokinetic parameters resumed in Table 2. This program approximates the blood kinetics to a bicompartimental model with two exponential

components: one of fast decay and another of slow decay.

Table 2. Calculated pharmacokinetics parameters for ^{177}Lu -BBNp6 in male *Balb-c* mice. Blood sample data was adjusted by two phases with an fast and a slow decay.

Pharmacokinetics Parameters	Value for ^{177}Lu -BBNp6
Equation	$C(t) = 820382.56^{-4.45t} + 7539.17^{-0.19t}$
$T_{1/2}$ fast phase (min)	9.6
$T_{1/2}$ slow phase (min)	222
* K_{12} (h^{-1})	0.71
** K_{21} (h^{-1})	0.23
*** K_{10} (h^{-1})	3.70
Distribution volume (mL)	28.66
Clearance ($\text{mL}\cdot\text{h}^{-1}$)	25.55
**** K_{ss} (h^{-1})	0.89

*Intravascular to extravascular space transfer constant; **Extravascular to intravascular space transfer constant; ***Intravascular space to excretion system transfer constant; ****Elimination rate constant

The biodistribution pattern of the radiolabeled bombesin derivative in *Nude* mice bearing PC-3 tumor xenografts are summarized in Fig. 5. Blood clearance was very rapid, as observed in pharmacokinetics studies. The studied bombesin derivative exhibited significantly tumor uptake in the first hour post injection.

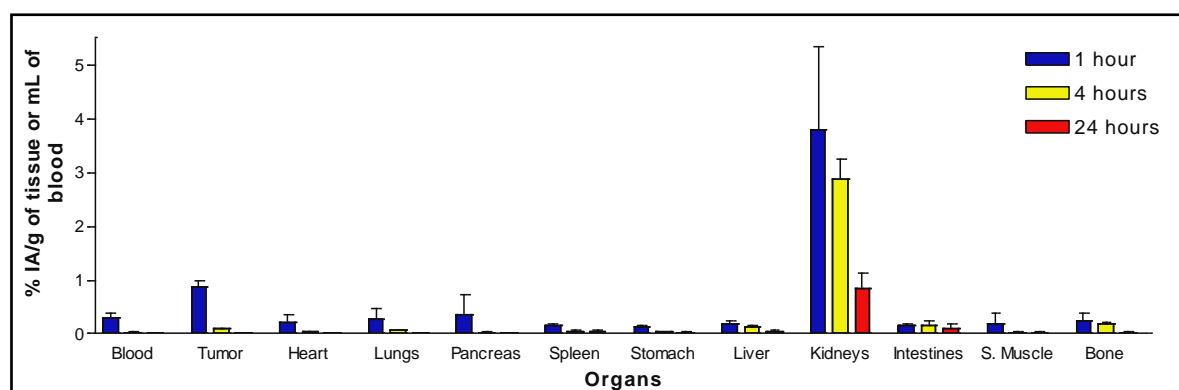


Figure 5. Biodistribution of ^{177}Lu -BBNp6 in male *Nude* mice bearing PC-3 tumor (n=3). Significant radioactivity could be detected in tumor and kidneys, indicating tumor target and renal excretion.

Scintigraphy images of radiolabeled bombesin derivative in normal *Balb-c* mice confirmed the results of the biodistribution assays and showed high kidneys uptake until 4 hours p.i., urinary excretion and low abdominal accumulation (Fig. 6).

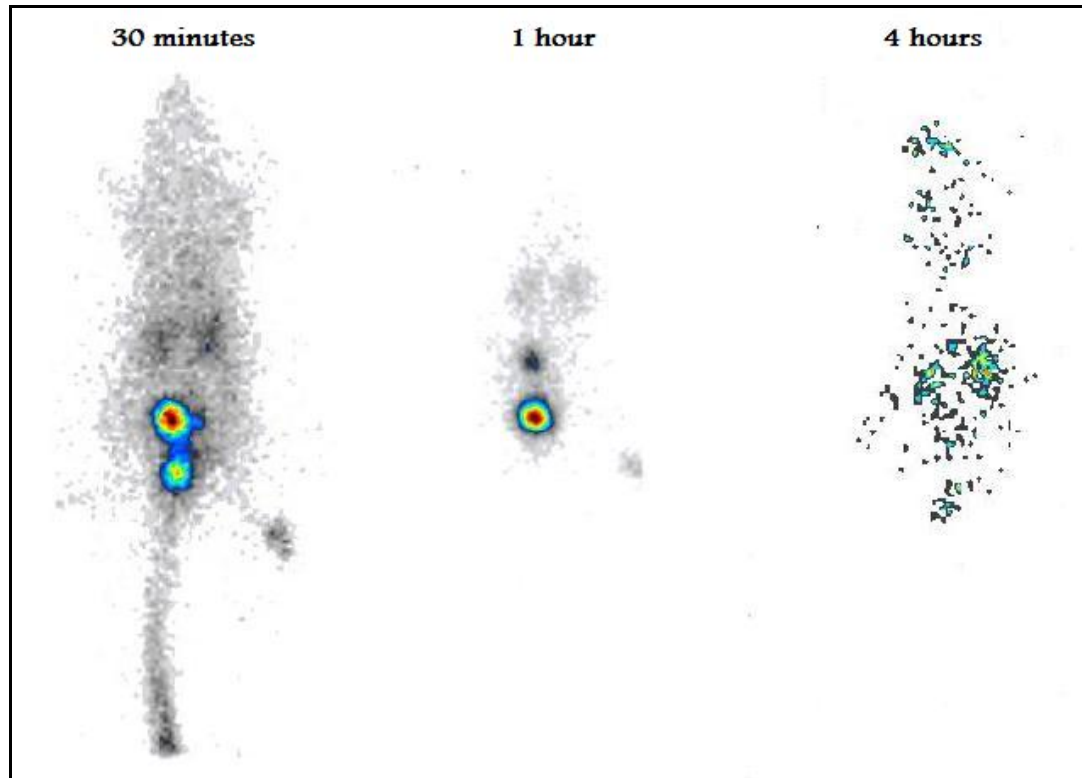


Figure 6. Scintigraphic static images of anesthetized *Balb-c* mice.

The images in *Nude* mice bearing PC-3 tumor xenografts showed important tumor uptake, especially 30 minutes and 1 hour post intravenous injection (Fig. 7). The region of interest (ROI) was calculated as the percentage of tumor region radioactivity compared to whole body radioactivity and was 10.9%, 8.21% and 4.51% 30 minutes, 1 and 4 hours p.i., respectively.

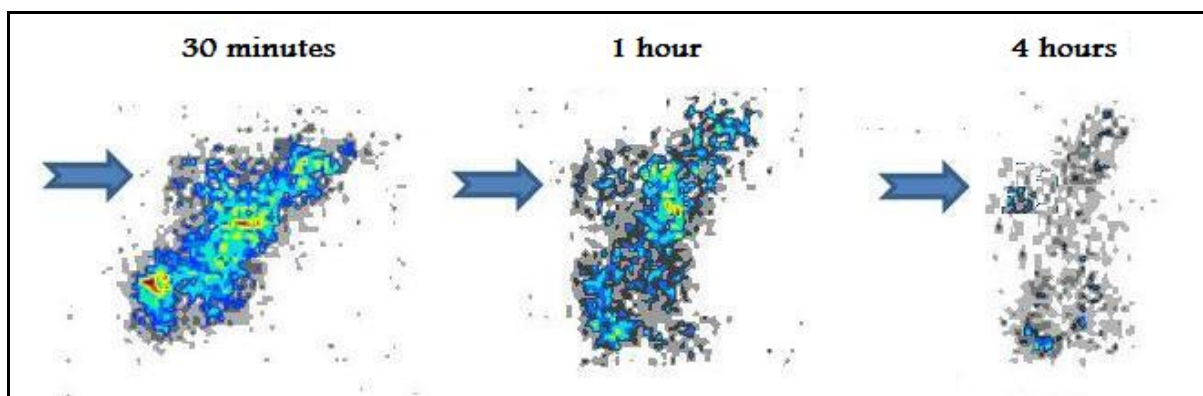


Figure 7. Scintigraphic static images of anesthetized *Nude* mice bearing PC-3 tumor.

4. DISCUSSION

Radiopharmaceuticals for systemic therapeutic applications are designed to deliver a therapeutic dose of radiation to specific disease sites. The ionizing radiation (e.g., α - or β -particles) given off from such compounds can either damage cellular components in the target tissue directly or indirectly via the free radicals. However, the potentially destructive properties of a therapeutic radioisotope emission are not limited to their cellular targets [12] and the efforts in developing novel bombesin derivatives are concentrated in reducing their binding to non-target tissues. This selectivity is influenced by the length and composition of the spacer group.

In this work we described the radiolabeling, chemical and biological properties of the novel bombesin analog BBNp6. In the developing procedure of this new derivative we intended to insert a hydrophilic spacer between the chelator group (DOTA) and the aminoacidic bombesin sequence 6-14. The results of pharmacokinetics analysis and biodistribution studies showed that ^{177}Lu -BBNp6 exhibits very rapid blood kinetic and fast renal excretion. So, the use of the six-aminoacid spacer group, in this case, influenced the biodistribution pattern of radiopeptide and determined low liver uptake and rapid clearance mainly performed by renal pathway.

An inconvenient of most studied bombesin analogs is their high *in vivo* uptake by pancreas and intestine due to the high density of GRP receptors in these mice tissues [6-10]. Although GRPr are found in rodent pancreas but rarely in human pancreas, these receptors are present in high density in human gastrointestinal mucosa [13] and gastrointestinal dosimetry would constitute a problem for the clinical application of radiolabeled bombesin derivatives in target radiotherapy. Biodistribution studies showed lower ^{177}Lu -BBNp6 uptake by pancreas than that described for other bombesin derivatives [6-10]. This lower pancreatic uptake of ^{177}Lu -BBNp6 could mean lower tumor affinity, but our results showed significantly prostate tumor uptake of this radiopeptide. This result is in accordance with some studies which have shown non linear relation between tumor uptake and pancreatic or intestinal uptake [7]. In addition, *in vivo* tumor uptake of ^{177}Lu -BBNp6 allowed PC-3 tumor detection by scintigraphy images, with the advantage of very low abdominal accumulation and background radiation in the images.

5. CONCLUSIONS

In this work we studied a novel bombesin derivative developed for prostate tumor imaging and treatment. The proposed bombesin derivative was easily radiolabeled with lutetium-177 and presented improved *in vitro* stability and *in vivo* properties. The derivative holds a high potential as radiopharmaceutical for human prostate tumor diagnosis and treatment.

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