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Preparation of [In-111]-labeled-DTPA-bombesin conjugates at high specific activity and stability: Evaluation of labeling parameters and potential stabilizers

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ABSTRACT

The aim of the present work was to obtain stabilized high specific activity (HSA) ^{111}In-labeled bombesin conjugates for preclinical evaluations. Parameters influencing the kinetics of labeling were investigated and the effect of stabilizers on HSA radiopeptides stability at room temperature were systematically categorized applying chromatography techniques. A SA of 174 GBq/μmol was achieved with high radiochemical purity, but the labeled compounds exhibited low stability. The addition of stabilizers avoided their radiolysis and significantly increased their stability.

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1. Introduction

Radiolabeled peptides have attracted considerable interest in nuclear medicine due to the specific binding to receptors overexpressed in some tumors, allowing the vizualization of fundamental biomolecular and cellular processes (Reubi and Maecke, 2008). Among the variety of peptides studied, bombesin apears as focus of interest.

Bombesin (BBN; Anastasi et al., 1970) is a 14-amino acid peptide analog of human gastrin releasing peptide (GRP) which binds specifically to gastrin-releasing peptide receptor (GRPr), predominantly expressed in prostate and breast cancer (Markwalder and Reubi, 1999; Gugger and Reubi, 1999). Several bombesin conjugates have been already synthesized and radiolabeled with different radioisotopes to be applied in tumor diagnosing by positron emission tomography (PET) or single photon emission tomography (SPECT) and/or therapy (Zhang et al., 2004; Lantry et al., 2006; Garayoa et al., 2007; Garrison et al., 2008; Liu et al., 2009; Lane et al., 2010; Honer et al., 2011; Wild et al., 2011).

In the development of radiolabeled bombesin conjugates, specific activity (SA) and stability are factors that should be considered. SA is an important quality control parameter and must be as high as possible to prevent receptor's saturation and physiological responses (Jensen et al., 2008). Low specific activity can compromise the uptake of the tracer by the tissue of interest *in vivo*, because the cold molecules must compete with radioactive ones for the binding sites, leading to lower target-to-background radioactivity ratio. On the other hand, very high specific activity can cause radiolysis, resulting in undesirable impurities due to oxidation, hydroxylation, aggregation and/or bond scission (Vallabhajosula et al., 2010; Chen et al., 2008). As these impurities can directly alter radiolabeled BBN derivatives biodistribution pattern in preclinical evaluations, radiolysis prevention is an important issue to consider in the development of high SA radiolabeled BBN conjugates and has not been extensively explored. In the present work, we describe the preparation of three new ^{111}In-DTPA-bombesin conjugates – BEYG$_3$, BEYG$_5$ and BEYG$_5$N – at high specific activity. We also characterize their time-course radiolytic degradation and compare the potential of known stabilizing agents in the preparation of high specific activity BBN conjugates for future preclinical and clinical studies.

2. Materials and methods

2.1. Chemicals

DTPA-bombesin conjugates were synthesized by piCHEM R&D (Austria). Identity and purity were confirmed by matrix-assisted

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Table 1
Amino acid sequences of bombesin and the three bombesin conjugates studied in this work.

Peptide	Chelator	Spacer	Amino acids													
			1	2	3	4	5	6	7	8	9	10	11	12	13	14
BBN			pGlu	Gln	Arg	Leu	Gly	Asn	Gln	Trp	Ala	Val	Gly	His	Leu	Met
$BEYG_3$	**DTPA**	–Tyr–Gly_3–						Asn	Gln	Trp	Ala	Val	Gly	His	Leu	Met
$BEYG_5$		–Tyr–Gly_5–						Asn	Gln	Trp	Ala	Val	Gly	His	Leu	Met
$BEYG_5N$		–Tyr–Gly_5–						Asn	Gln	Trp	Ala	Val	Gly	His	Leu	Nle

laser desorption/ionization mass spectroscopy and reverse-phase high performance liquid chromatography (RP-HPLC). The amino acid sequence of the three studied peptides is compared to bombesin in the Table 1.

Indium-111 chloride ($^{111}InCl_3$) in 0.05 M HCl was purchased from Nordion (Canada). All other reagents were purchased from Merck (Germany), with the following exceptions: trifluoroacetic acid (TFA), gentisic acid and methionine were from Sigma–Aldrich (USA) and Chelex 100 ion exchange resin was from BioRad (USA). All reagents were of analytical grade and the solvents for HPLC were HPLC grade.

2.2. Analytical methods

2.2.1. Thin layer chromatography (TLC)

Thin layer chromatography in silica gel 60 (TLC-SG, Merck, Germany) was applied to determine free indium in radiolabeling mixtures, with ethylenediaminetetraacetic acid (EDTA, 0.2 M, pH 5.0) as solvent. R_f of ^{111}In-DTPA-bombesin conjugates was 0.0–0.2 and R_f of free indium was 0.6–0.8.

2.2.2. High performance liquid chromatography (HPLC)

Reversed-phase high-performance liquid chromatography (RP-HPLC) analyzes of $^{111}InCl_3$ and radiolabeled and non-radiolabeled DTPA-bombesin conjugates were performed on a Shimadzu system (Japan) equipped with an analytical reversed-phase C-18 column (4.0 × 150 mm, 5 μm – Waters, USA); a SPD-10A UV–vis (Shimadzu, Japan) absorbance detector (λ=280 nm); a CTO-10 Avp column heater (Shimadzu, Japan) and a radiometric in-line Shell Jr. 1000/2000 (Shell-usa, USA) NaI solid scintillation detector. Two different methods were used (Hu et al., 2002), applying trifluoroacetic acid 0.1% in water (A) and trifluoroacetic acid 0.1% in acetonitrile (B) as solvents.

Method 1. Flow rate, 1.5 mL/min; linear gradient, 10 to 90% solvent B in 15 min and change back to 10% solvent B for 5 min.

Method 2. Flow rate, 1.0 mL/min; linear gradient, 10 to 40% solvent B in 20 min and change back to 10% solvent B for 5 min.

2.3. Development of a method for radiolabeling DTPA-bombesin conjugates with ^{111}In at high specific activity

The optimization of ^{111}In-labeling parameters were performed with the bombesin conjugate $BEYG_5$. All reagents were prepared with Chelex 100 treated metal free water.

2.3.1. Study of radiolabeling conditions

The labeling conditions were investigated for the peptide $BEYG_5$, in 200 μL of final volume of sodium acetate buffer 0.4 M at pH 4.5. The studied parameters were temperature (25–55 °C), peptide mass (0.625–10 μg), $^{111}InCl_3$ activity (18.5–1110 MBq) and time of reaction (5–30 min). The radiochemical purity and peptide integrity of the preparations were determined by TLC and HPLC, as described earlier.

2.3.2. Methionine concentration

$BEYG_5$ was radiolabeled according to optimized parameters to determine the methionine concentration that should be added to the mixtures to avoid methionine residue oxidation. The radiolabeling reaction was performed in the absence and in the presence of methionine amino acid (0.5 mg/mL or 5 mg/mL in sodium acetate buffer 0.4 mol/L pH 4.5). The radiochemical purity of the preparations was determined by TLC and HPLC (method 1).

2.4. Radiolabeling of $BEYG_3$, $BEYG_5$ and $BEYG_5N$ with ^{111}In

The bombesin conjugates were radiolabeled considering the optimized parameters determined in the experiments described earlier. The radiochemical purity of the preparations was determined by TLC.

The stability of the radiopeptides was analysed by HPLC (method 2), immediately after the end of the reactions, in the absence and presence of methionine, to evaluate the formation of oxidized species at different conditions.

2.5. Stability of ^{111}In-DTPA-bombesin conjugates at room temperature: Comparative evaluation of potential stabilizers

To compare the effect of potential stabilizers on *in vitro* stability of labeled peptides, the stabilizer was added to the preparations immediately after radiolabeling in the presence of methionine and they were stored at room temperature for 24, 48 and 72 h, followed by TLC and HPLC analysis to determine radiochemical purity and the percentage of radiolytic degradants. Three stabilizers were evaluated: ascorbic acid (AA), gentisic acid (GA) and ethanol (EtOH). A solution of each potential stabilizer was prepared in NaCl 0.9% pH 7.4 or 10% (v/v) for ethanol (Chen et al., 2008). The ^{111}In-DTPA-bombesin conjugates preparations were mixed with the stabilizer solution (500 μL) to yield a radioactivity concentration of 0.3 GBq/mL and a final stabilizer concentration of 10 mg/mL or 10% v/v for the EtOH. For comparison, the radiochemical purity and HPLC profiles of non-stabilized preparations (without methionine and with methionine 0.5 mg/mL only) after storing at room temperature for 24, 48, and 72 h were also analyzed.

2.6. Statistical analysis

The results are expressed as Mean (Range) for $n=2$ and Mean ± SD for $n \geq 3$. Statistical analysis of the results was performed using an unpaired, 2-tailed t test (Excel, Microsoft). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Development of a method for radiolabeling DTPA-bombesin conjugates with ^{111}In at high specific activity

The radiochemical purity of the preparations, determined by TLC, after radiolabeling of $BEYG_5$ with indium-111 (20+2 MBq) at

different temperatures is shown in Table 2. The radiopeptide was obtained at high radiochemical purity in all temperatures analyzed and the temperature chosen for further experiments was 25 °C. Although the resistance of bombesin derivatives and other small peptides to high temperatures has already been demonstrated (Breman et al., 2002; Pujatti et al., 2011), radiolabeling at room temperature promotes less damage to peptides integrity and assures practical routine applicability and radiation protection requirements to the operator and to the environment. Despite of their lower stability, the possibility of radiolabeling at room temperature is the main advantage of DTPA-chelated to DOTA-chelated compounds (Breman et al., 2002; Schroeder et al., 2010).

After determining the temperature of the reaction as 25 °C, different $BEYG_5$ mass (0.625–10 μg) were radiolabeled using 20+2 MBq of radionuclide and the results are shown in Fig. 1. High radiochemical purity (>95%) were achieved when 2.5 (95.3 ± 0.3%), 5 (96.3 ± 0.7%) and 10 μg (98.8 ± 1.0%) of peptide reacted with indium-111, but the radiochemical purity was significantly higher when 10 μg ($p<0.05$) of peptide was applied in the reaction. The mass of peptide chosen for further optimization was 10 μg.

The condition applying 10 μg of peptide, 20+2 MBq of ^{111}In and 25 °C was used to optimize the time of reactions. Times varying from 5 to 30 min were studied and the radiochemical purity was higher than 95% at all times analyzed (Table 3). The intermediate time (15 min) was applied in further reactions.

The effect of increasing radionuclide activities on radiochemical purity of $[^{111}In]BEYG_5$ is presented on Table 4. The reactions were performed with 10 and 2.5 μg of peptide, the lower peptide mass that produces a radiochemical purity higher than 95% was achieved (Fig. 1), and ^{111}In-activity varying from 18.5 to 1111 MBq (0.5–30 mCi). Higher radiochemical yields (>95%) were obtained with the peptide mass of 10 μg and 18.5 to 925 MBq of indium-111, producing a maximum specific activity peptide of 174 GBq/μmol.

Table 2
Influence of radiolabeling temperature on the radiochemical purity of $[^{111}In]BEYG_5$. $BEYG_5$ (10 μg) reacted with indium-111 (20+2 MBq) for 30 min at different temperatures, pH 4.5 and 200 μg of final volume. The radiochemical purity was determined by TLC (n=2).

Radiolabeling temperature (°C)	Radiochemical purity (%)
25	98.8 (0.4)
40	98.8 (1.6)
55	96.6 (1.8)

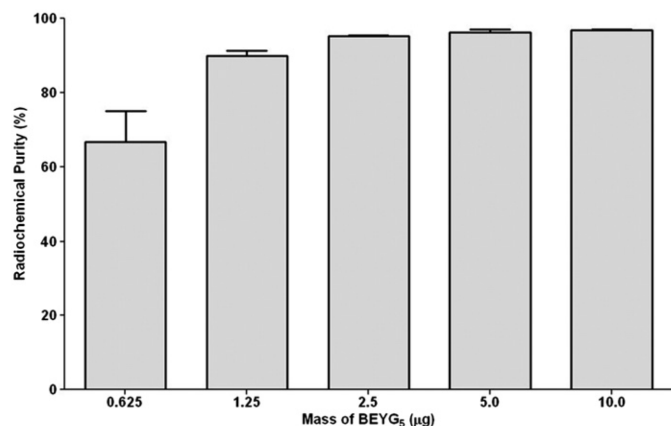

Fig. 1. Radiochemical purity of $[^{111}In]BEYG_5$ depending on the peptide mass. The reactions were performed with 20 ± 2 MBq of $^{111}InCl_3$, at 25 °C for 30 min and a final volume of 200 μL (n=3). The radiochemical purity was 66.6 ± 8.5, 89.9 ± 1.3, 95.30 ± 0.7, 96.3 ± 0.7 and 98.8 ± 1.0% when 0.625, 1.25, 2.5, 5.0 and 10 μg of the peptide reacted with ^{111}In, respectively.

Table 3
Influence of time of reaction on the radiochemical purity of $[^{111}In]BEYG_5$. $BEYG_5$ (10 μg) reacted with indium-111 (20+2 MBq) at 25 °C, pH 4.5 and 200 μg of final volume for different times. The radiochemical purity was determined by TLC (n=2).

Time of reaction (minutes)	Radiochemical purity (%)
5	96.8 (1.0)
10	97.0 (1.4)
15	97.7 (3.0)
20	97.9 (0.4)
25	97.1 (2.0)
30	97.1 (0.8)

The HPLC profiles (method 1) of $[^{111}In]BEYG_5$ obtained at different specific activities are shown in Fig. 2. The retention time (RT) of $[^{111}In]BEYG_5$ was 6.70 ± 0.02 min and no significant $^{111}InCl_3$ (RT 1.05 ± 0.05 min; not shown) contamination was detected in the labeling mixtures.

HPLC analysis also revealed a second peak of RT 5.77 ± 0.04 min in some chromatograms of $[^{111}In]BEYG_5$. Under those radiolabeling conditions, there were some discrepancies observed between the radiochemical purity calculated by HPLC and TLC (Table 4), probably due to the incapability of the last to separate this radiodegradant from the intact radiopeptide. Chen et al. (2008) described this second peak as the methionine-oxidized peptide for the bombesin derivative $[^{177}Lu]$AMBA. This oxidized form has already been described for other radiolabeled bombesin analogs as a radiolytic degradant or a product of the radiolabeling in an acid pH and its formation can be avoided by both the addition of an antioxidant agent or the replacement of the terminal methionine (Met) by a synthetic amino acid, such as norleucine (Nle) (Maina et al., 2005; Zhang et al., 2007; Chen et al., 2008).

In order to determine if the second peak observed in some radiochromatograms of $[^{111}In]BEYG_5$ is due to methionine oxidation, the peptide was radiolabeled at optimized conditions (sodium acetate buffer 0.4 mol/L pH 4.5) and in the absence or presence of methionine amino acid (0.5 mg/mL or 5 mg/mL), followed by HPLC analysis. Methionine did not alter significantly the percentage of the species in the product. This impurity represents only 2 to 4% of the total product. In addition, the excess of methionine increased the percentage of free radionuclide in the preparation (Retention Time=1.20 ± 0.05 min) and decreases the radiochemical purity to 91.9 ± 0.6%.

The optimized parameters determined for labeling $BEYG_5$ with ^{111}In were: 15 min of reaction at 25 °C, 10 μg of peptide and pH 4.5. At those conditions, a maximum radionuclide to peptide ratio of 174 GBq/μmol was achieved. These conditions did not promote $BEYG_5$ methionine amino acid oxidation.

3.2. Radiolabeling of $BEYG_3$, $BEYG_5$ and $BEYG_5N$ with ^{111}In

The bombesin conjugates $BEYG_3$ and $BEYG_5N$ and also $BEYG_5$ were radiolabeled applying the optimized parameters in the absence and in the presence of methionine 0.5 mg/mL and the radiochemical purities achieved, determined by TLC, are shown in Table 5. It can be observed that the optimized parameters were successfully applied to the other two bombesin conjugates and a high radiochemical purity was obtained.

The HPLC profile (method 2) of the three radiopeptides produced at optimized conditions are presented in the Fig. 3. The high radiochemical purity, determined by TLC, was confirmed by HPLC analysis, in which a low amount of free indium-111 was

Table 4
Influence of ^{111}In activity on the radiochemical purity of [^{111}In]BEYG$_5$. BEYG$_5$ (2.5 or 10 μg) reacted with different ^{111}In activities at 25 °C, pH 4.5 and 200 μL of final volume for 15 min.

Mass of BEYG$_5$ (μg)	^{111}InCl$_3$ activity (MBq/mCi)	Radiochemical purity (TLC) of [^{111}In]BEYG$_5$ (%)	Radiochemical purity (HPLC) of [^{111}In]BEYG$_5$ (%)	Radionuclide activity: peptide mass ratio (MBq/μg)	Specfic activity of [^{111}In]BEYG$_5$ (GBq/μmol)	n
2.5	18.5/0.5	95.3 ± 0.3	Nd	7.4	13.9	3
2.5	74/2.0	92.7 ± 0.3	Nd	29.6	55.6	2
2.5	111/3.0	84.0 (2.8)	Nd	44.4	83.4	2
2.5	148/4.0	83.1 (0.8)	Nd	59.2	111.2	2
10	18.5/0.5	96.9 ± 0.1	98.4 ± 0.3	1.85	3.5	3
10	37/1.0	96.7 (0.4)	97.5 (0.8)	3.7	6.9	2
10	55.5/1.5	96.7 (1.0)	Nd	5.55	10.4	2
10	74/2.0	97.6 ± 0.8	99.0 ± 0.4	7.4	13.9	3
10	92.5/2.5	96.9 ± 0.2	Nd	9.25	17.4	3
10	111/3.0	97.2 ± 0.6	93.3 ± 7.2	11.1	20.9	3
10	148/4.0	97.5 (0.4)	98.9 (1.0)	14.8	27.8	2
10	185/5.0	97.9 (2.4)	98.1 (2.0)	18.5	34.8	2
10	296/8.0	97.7 (0.4)	98.0 (1.2)	29.6	55.6	2
10	555/15.0	95.2 (0.2)	Nd	55.5	104.3	2
10	832.5/22.5	96.0 (0.8)	Nd	83.25	156.5	2
10	925/25.0	97.2 (2.0)	96.4 (0.8)	92.5	174.0	2
10	1110/30.0	92.3 (1.8)	Nd	111.0	209.0	2

nd – not determined.

identified (RT 1.67 ± 0.05 min). In addition, the chromatograms also reveled two other species with shorter retention time than the main specie (9.12 and 12.22 min). These two species did not appear on the HPLC profile of the three unlabeled peptides applying UV/visible detector (Fig. 4) and their appearance was not avoided by the presence of methionine in radiolabeling medium (Fig. 3).

The specie with RT of 12.22 min was observed in both HPLC profiles of [^{111}In]BEYG$_3$ and [^{111}In]BEYG$_5$, which present the methionine residue, but not in HPLC profile of [^{111}In]BEYG$_5$N, in which the methionine was replaced by norleucine. Although methionine could not avoid the formation of other species during labeling, this result suggests that the presence of terminal methionine influences the appearance of this specie.

3.3. *Stability of ^{111}In-DTPA-bombesin conjugates at room temperature: Comparative evaluation of potential stabilizers*

The effect of stabilizers on ^{111}In-labeled bombesin conjugates were evaluated at room temperature by TLC (Table 6) and HPLC (Table 7) for 24, 48 and 72 h by adding AA, EtOH or GA at the end of the reactions. Differences in TLC and HPLC values for radiochemical purity were observed in all times analyzed probably due to the incapacity of TLC to separate the radiolysis products. These radiodegradants probably migrate with the same R_f of the intact radiopeptides.

The HPLC profile of [^{111}In]BEYG$_3$, [^{111}In]BEYG$_5$ and [^{111}In]BEYG$_5$N stored in the absence and presence of each stabilizer (not shown) suggests that the three radiolabeled bombesin conjugates are degradated to species with intermediary retention times in the absence of stabilizers, especially after 72 h of storage at room temperature. It can also be observed that the bombesin conjugate [^{111}In]BEYG$_3$ presents higher stability than [^{111}In]BEYG$_5$ and [^{111}In]BEYG$_5$N in the absence of stabilizing agents or presence of methionine 0.5 mg/mL. Moreover, the specie with retention time of 12.22 min, observed as a product from radiolabeling reaction, is the major radiolytic degradant in the absence of stabilizers.

AA, EtOH and GA had significant stabilizing effect at the concentration tested and prevented all damage, except the appearance of the specie with retention time of 12.22 min. Almost no other degradants were observed during three days of storage at room temperature. The specie with RT 12.22 min was not observed as a radiodegradant of [^{111}In]BEYG$_5$N, suggesting that the presence of this specie is related to the terminal methionine. After 72 h of storage, it was found that GA present a higher stabilizer effect for [^{111}In]BEYG$_3$, AA present a higher stabilizer effect for [^{111}In]BEYG$_5$ and EtOH present a higher stabilizer effect for [^{111}In]BEYG$_5$N. However, after 24 h of storage, a higher stabilizer effect was observed with EtOH for the three bombesin conjugates.

4. Discussion

There has been great current interest in radiolabeled small peptides as diagnostic and therapeutic radiopharmaceuticals. In developing a new-receptor based radiopharmaceutical, several factors need to be considered to satisfy the clinical need and national regulations. The radiopharmaceutical must demonstrate efficacy, favorable pharmacokinetics and high target-to-backgroud ratio. For that, it must have high radiochemical purity (>90%), high specific activity and high stability, to retain its chemical and biological integrity during release and transportation (Liu and Ellars, 2001; Vallabhajosula et al., 2010).

In this work, three new ^{111}In-DTPA-chelated bombesin conjugates were prepared and stabilized, in order to obtain high quality radiopharmaceuticals for preclinical evaluations. The three peptides were radiolabeled with indium-111 at high radiochemical purity and high specific activity (174 GBq/μmol). The radiolabeling reaction was completed after 15 min at 25 °C. Similar results were already described for other bombesin derivatives (Breman et al., 2002; Ho et al., 2009; Schroeder et al., 2010). However, in contrast to some literature data, [^{111}In]BEYG$_3$, [^{111}In]BEYG$_5$ and [^{111}In]BEYG$_5$N did not present high stability in saline solution at room temperature (Breman et al., 2002; Ho et al., 2009).

Radiopharmaceuticals comprising α- or β-emitting radionuclides often undergo radiolysis during preparation and storage. During radiolysis, emissions from the radionuclide attack the metal chelate, targeting biomolecule and other compounds in proximity, which results in decomposition or destruction of the metal chelate or/and the biomolecule (Liu et al., 2003). Although these radiolytic effects are more remarkable for therapeutic radioisotopes, most diagnostic radionuclides, including indium-111, emit not only photons, but also Auger electrons, which can

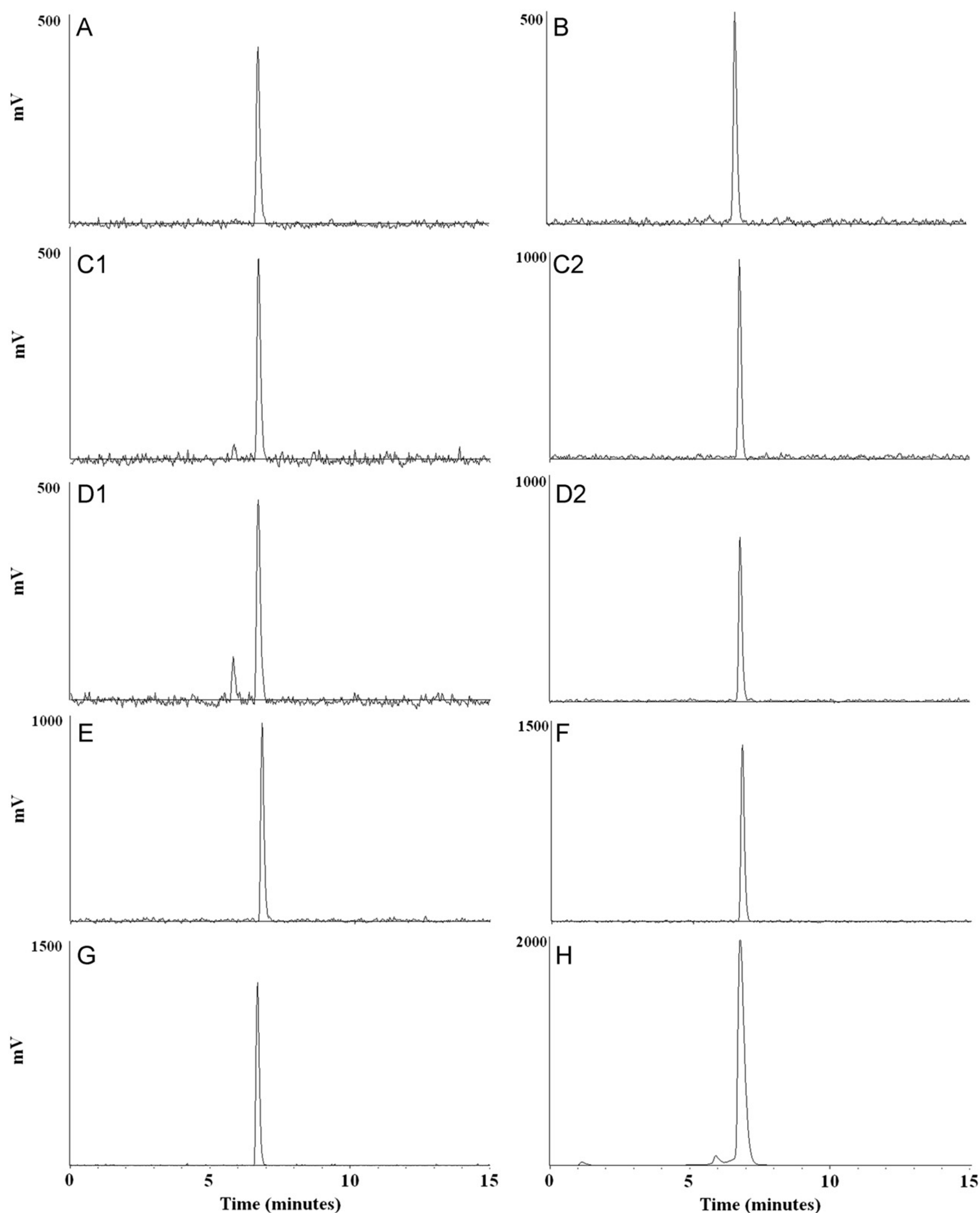

Fig. 2. Representative reversed phase HPLC profile (C-18 column 4.0 × 150 mm, 5 μm; radioactive detector; method 1) of [^{111}In]BEYG$_5$ obtained at 3.5 (A); 6.9 (B); 13.9 (C1 and C2); 20.9 (D1 and D2); 27.8 (E); 34.8 (F); 55.6 (G) and 174 (H) GBq/μmol of specific activity. The numbers 1 and 2 in some letters indicate profiles obtained in different days. The retention time (RT) of [^{111}In]BEYG$_5$ and ^{111}InCl$_3$ were 6.70 ± 0.02 min and 1.05 ± 0.05 min, respectively.

have particle effects. Since *in vivo* biodistribution of bombesin conjugates is largely dependent on the binding to receptors in tumor cells, radiolytic degradation may lead to unwanted radiation to normal tissues and failure of diagnostic procedure. In preclinical studies, radiolytic degradation can cause unreliable results. Therefore, there is a need for a stabilizer to stabilize the radiolabeled bombesin conjugates.

A large number of stabilizers have already been described to prevent radiolytic damage in radiopharmaceuticals formulations. These studies have demonstrated that compounds such as AA, GA

and methionine are effective radiostabilizers in some applications (Chen et al., 2008; Liu and Ellars, 2001; Liu et al., 2003). However, these stabilizers, as single agents, could not fully protect [^{111}In]BEYG$_3$, [^{111}In]BEYG$_5$ and [^{111}In]BEYG$_5$N from radiolytic degradation, but they significantly increased their stability at room temperature up 72 h of storage ($p < 0.001$).

The degree of radiolytic damage observed is expected to be dependent upon radioconcentration, specific activity and the type of ionizing radiation used. In addition, the effectiveness of a particular stabilizer also depends on the sensitivity of various functional groups in the targeting molecule itself (Chen et al., 2008). For example, despite of the similarity among their structures, after 72 h of storage, GA presented a higher stabilizer effect for [^{111}In]BEYG$_3$, AA presented a higher stabilizer effect for [^{111}In]BEYG$_5$ and EtOH presented a higher stabilizer effect for [^{111}In]BEYG$_5$N. These differences were not observed after 24 h of storage, when higher stabilizer effect was observed with EtOH for the three bombesin conjugates. In addition, [^{111}In]BEYG$_3$ presented a significantly ($p < 0.05$) higher stability than [^{111}In]BEYG$_5$ and [^{111}In]BEYG$_5$N in all conditions and times analyzed and, in contrast to the results obtained for these last two radiopeptides, methionine presented a significantly stronger stabilizer effect for [^{111}In]BEYG$_3$. So, small changes on the spacer of bombesin conjugates promoted considerable differences in the stability of labeled compounds. These differences can influence in their biological behavior.

Oxidized methionine residue has been described as the major radiolytic degradant of bombesin derivatives and the targeting capability can be totally inactivated by this oxidation (Chen et al., 2008). The results of the present work showed a minimal degradation of the radiopeptides during radiolabeling, in which a specie with a retention time of 12.22 min was identified as the major degradant (Fig. 4). This specie was also observed as

Table 5
Radiochemical purity of the three bombesin conjugates (174 GBq/μmol) radiolabeled according the optimized parameters. The radiochemical was determined by TLC ($n=5$).

Bombesin conjugate (%)	Molecular weight (g/mol)	Radiochemical purity	
		Without methionine	With methionine (0.5 mg/mL)
[^{111}In]BEYG$_3$	1765.0	97.3 ± 0.8	96.1 ± 1.3
[^{111}In]BEYG$_5$	1879.1	97.2 ± 1.0	96.1 ± 0.5
[^{111}In]BEYG$_5$N	1858.8	97.8 ± 0.1	97.6 ± 0.4

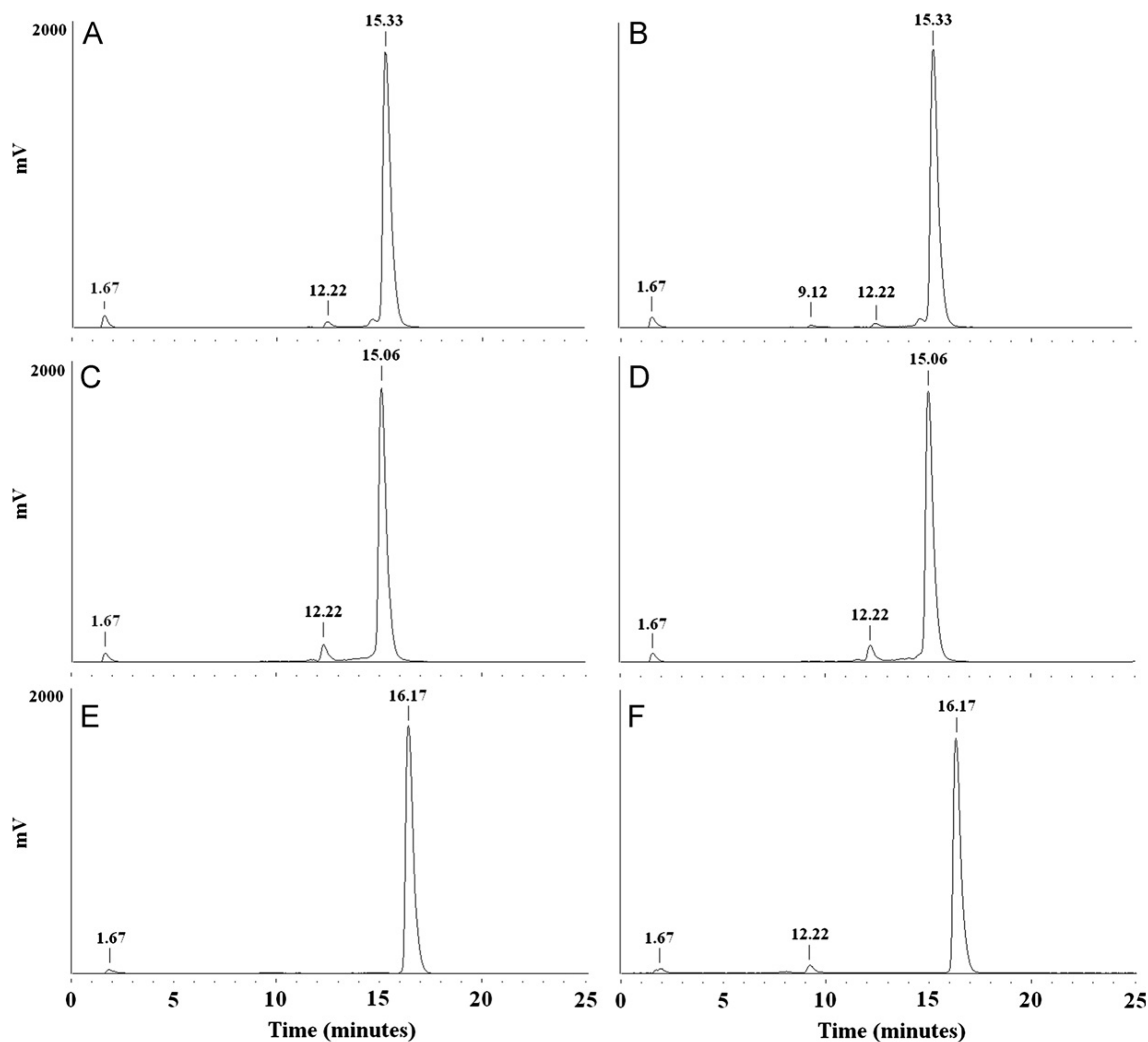

Fig. 3. Representative reversed phase HPLC profile (C-18 column 4.0 × 150 mm, 5 μm; radioactive detector; method 2) of radiolabeled bombesin conjugates (174 GBq/μmol) [^{111}In]BEYG$_3$ (A and B), [^{111}In]BEYG$_5$ (C and D) and [^{111}In]BEYG$_5$N (E and F) produced in the absence (A, C and E) and presence of methione 0.5 mg/mL (B, D and F). The numbers in the figures represent the retention times, in minutes, of each species.

Table 6
Effect of different stabilizer agents on radiolabeled bombesin conjugates stability. The high specific activity ^{111}In-labeled bombesin conjugates were stored in the absence or presence of different stabilizer agents at room temperature for different times. The radiochemical purity was determined by TLC ($n=3$).

Storage condition	Radiochemical specie with R_f 0.0–0.1 (%)								
	[^{111}In]BEYG$_3$			[^{111}In]BEYG$_5$			[^{111}In]-BEYG$_5$N		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
Without stabilizer	92.7 ± 0.6	82.4 ± 0.7	82.8 ± 0.1	92.3 ± 1.1	93.3 ± 5.7	82.0 ± 3.5	85.8 ± 1.1	79.0 ± 1.3	64.3 ± 5.2
Methionine 0.5 mg/mL	95.9 ± 0.3	92.7 ± 0.8	92.4 ± 0.7	95.4 ± 0.6	88.1 ± 0.4	84.5 ± 1.1	91.9 ± 4.9	90.5 ± 3.1	85.6 ± 1.3
Ascorbic acid (10 mg/mL)	96.6 ± 0.2	95.0 ± 0.1	94.6 ± 0.2	94.6 ± 0.6	94.9 ± 1.1	95.8 ± 0.5	96.1 ± 0.1	92.9 ± 0.1	89.6 ± 0.2
Ethanol (10% v/v)	95.8 ± 0.6	95.6 ± 0.5	94.5 ± 3.1	97.1 ± 0.1	94.9 ± 0.7	96.7 ± 0.1	97.7 ± 0.2	97.7 ± 0.2	95.7 ± 0.5
Gentisic acid (10 mg/mL)	97.3 ± 0.1	93.9 ± 0.8	93.6 ± 0.1	95.9 ± 1.6	97.1 ± 0.8	93.4 ± 0.5	90.3 ± 0.8	86.6 ± 2.5	78.5 ± 4.0

Table 7
Effect of different stabilizer agents on radiolabeled bombesin conjugates stability. The high specific activity ^{111}In-labeled bombesin conjugates were stored in the absence or presence of different stabilizer agents at room temperature for different times. The radiochemical purity was determined by HPLC ($n=3$).

Storage condition	Radiochemical specie with RT correspondent to the labeled bombesin conjugate (%)								
	[^{111}In]BEYG$_3$			[^{111}In]BEYG$_5$			[^{111}In]BEYG$_5$N		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
Without stabilizer	95.9 ± 1.0	0.00	0.00	21.6 ± 0.2	0.2 ± 0.1	0.00	17.0 ± 1.2	1.3 ± 0.2	0.00
Methionine 0.5 mg/mL	99.0 ± 0.8	86.1 ± 1.2	75.98 ± 0.9	18.9 ± 0.4	0.3 ± 0.1	0.00	69.6 ± 0.6	37.7 ± 0.3	15.1 ± 1.8
Ascorbic acid (10 mg/mL)	95.9 ± 0.4	90.3 ± 0.3	83.11 ± 1.1	88.6 ± 1.3	75.0 ± 0.5	74.6 ± 0.7	89.8 ± 1.0	83.2 ± 1.4	75.1 ± 0.7
Ethanol (10% v/v)	97.6 ± 0.6	96.3 ± 0.4	82.16 ± 0.3	91.7 ± 0.3	83.3 ± 0.9	70.2 ± 1.0	94.4 ± 0.8	88.5 ± 0.9	88.7 ± 1.9
Gentisic acid (10 mg/mL)	95.5 ± 0.7	93.9 ± 2.5	90.89 ± 0.3	75.1 ± 0.5	58.0 ± 1.2	47.6 ± 0.5	78.9 ± 1.7	72.7 ± 1.2	54.7 ± 2.8

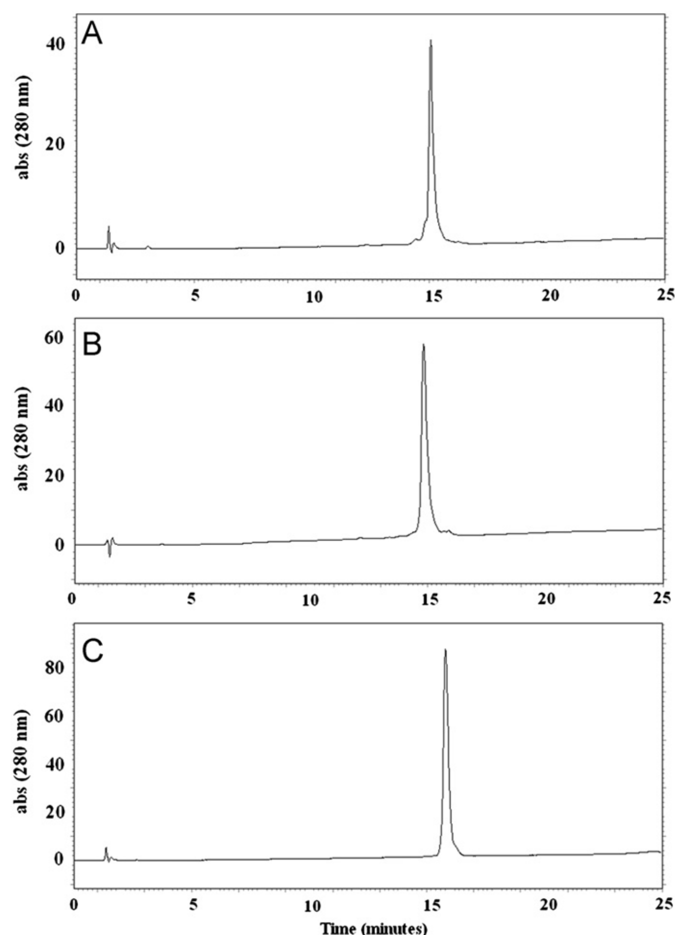

Fig. 4. Representative reversed phase HPLC profile (C-18 column 4.0 × 150 mm, 5 µm; UV detector; method 2) of unlabeled bombesin conjugates BEYG$_3$ (A), BEYG$_5$ (B) and BEYG$_5$N (C).

the main radiolysis product of [^{111}In]BEYG$_3$ and [^{111}In]BEYG$_5$ in AA, EtOH and AG stabilized preparations. Although the formation of this specie was not avoided by the presence of methionine during radiolabeling, it was not observed in [^{111}In]BEYG$_5$N preparations and stabilized solution. Because the methionine amino acid in [^{111}In]BEYG$_3$ and [^{111}In]BEYG$_5$ was replaced by norleucine [^{111}In]BEYG$_5$N, the specie with RT of 12.22 min is probably related to methionine amino acid and can represent the oxidized species. In this case, methionine alone was insufficient for [^{111}In]BEYG$_3$ and [^{111}In]BEYG$_5$ protection and could be replaced by selenium methionine, as already described by Chen et al. (2008). Despite of the presence of this degradant product (RT 12.22 min), it represents only a small amount of total radioactivity after radiolabeling and 24 h of storage in some stabilized preparations, which kept their radiochemical purity higher than 90%, suitable for preclinical evaluations.

5. Conclusion

This study describes the preparation and stabilization of three ^{111}In-labeled-bombesin conjugates. By using methionine aminoacid during radiolabeling and ethanol (10%), gentisic acid (10 mg/mL) or ascorbic acid (10 mg/mL) in saline solution to dilute and stabilize the reaction after metal incorporation, a high radiochemical purity (> 90%) was obtained and maintened for at least 24 h. The degree of stabilizing depends upon peptide's structure. These preparations will be used for preclinical comparative evaluations of the three bombesin conjugates.

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