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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.

1. Introduction

Radiolabeled peptides have attracted considerable interest in nuclear medicine due to the specific binding to receptors over-expressed 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 $BEYG_5N$ – 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