Applied Radiation and Isotopes 70 (2012) 2440–2445

Contents lists available at SciVerse ScienceDirect

Applied Radiation and Isotopes

journal homepage: www.elsevier.com/locate/apradiso

Kit formulation for ^{99m}Tc-labeling of HYNIC-βAla-Bombesin$_{(7-14)}$

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HIGHLIGHTS

- Bombesin kit formulations were successfully prepared.
- High radiochemical purity was achieved for $KBBN_{50}$ and $KBBN_{100}$ formulations.
- *In vitro* assays demonstrated high storage stability for $KBBN_{50}$.
- Biodistribution and scintigraphic images corroborate with *in vitro* results.

ARTICLE INFO

Article history:
Received 19 December 2011
Received in revised form
18 June 2012
Accepted 21 June 2012
Available online 28 June 2012

Keywords:
Kit formulation
Freeze-drying/lyophilization
Radiolabeling
Bombesin
Technetium-99m

ABSTRACT

Bombesin (BBN) is a tetradecapeptide that binds specifically to gastrin-releasing peptide receptors. Several forms of cancer, including lung, prostate, breast, and colon express receptors for bombesin-like peptides. Radiolabeled BBN analogs with a high affinity for these receptors might be used for scintigraphic imaging. Kit formulations for labeling these molecules are important for routine preparation. A freeze-dried kit for labeling HYNIC-βAla-Bombesin$_{(7-14)}$ with technetium-99m was prepared, and its storage stability was evaluated by *in vitro* and *in vivo* assays.

1. Introduction

Cancer imaging techniques using radiotracers targeted to specific receptors have yielded successful results demonstrating the utility of such approaches for developing specific radiopharmaceutics (Santos-Cuevas et al., 2009). Small receptors-binding peptides are currently the agents of choice for receptor imaging and tumor targeting (Faintuch et al., 2008). *In vivo* functional imaging techniques can help to diagnose and stage tumors, optimize drug scheduling, and predict response to a therapeutic modality, which would be advantageous to both patient and oncologist (Conti et al., 1996; Chen et al., 2006; de Barros et al., 2010a).

Regulatory peptide receptors are over-expressed in numerous human cancer cells. These receptors have been used as molecular targets for radiolabeled peptides to locate tumors. In recent years, many studies have been performed to identify peptide analogs able to target these tumors, such as gastrin-releasing peptide, somatostatin, neurotensin, vasoactive intestinal peptide, etc. (Okarvi and Al-Jammaz, 2003; Zhang et al., 2007; Koopmans et al., 2009; Virgolini et al., 2009; Ambrosini et al., 2011; Dalmo et al., 2012; Däpp et al., 2011).

Bombesin (BBN) is a tetradecapeptide isolated from the skin of the European frog *Bombina bombina* (Anastasia et al., 1971). The mammalian counterpart is the 27 amino acid gastrin-releasing peptide (GRP). A variety of tumors have been found to express receptors for this peptide, such as lung, prostate, breast, pancreas and colon (Reubi, 2003). Radiolabeled BBN analogs with a high affinity for these receptors might therefore be used for scintigraphic imaging of these tumor types (Smith et al., 2005; Jensen et al., 2008). Technetium-99m is a very cost-effective isotope which can be easily obtained from a ^{99}Mo/^{99m}Tc generator (de Barros et al., 2009). Some chelating approaches have been used to stabilize the radioactive core after *in vivo* administration, such as N_3S (Okarvi and Jammaz, in press), N_2S_2 (Santos-Cuevas et al., 2011), carbonyl (Ferro-Flores et al., 2010), HYNIC (Ananias et al., 2011). 2-Hydrazinonicotinamide (HYNIC) is an attractive bifunctional chelating ligand for preparing ^{99m}Tc-labeled peptides (Surfraz et al., 2007; Guo et al., 2011; Lu et al., 2011) because it shows a high labeling efficiency and

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0969-8043/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.apradiso.2012.06.022

its usage with various co-ligands (e.g., ethylendiaminediacetic acid (EDDA), tricine) allows for easy modification of the hydrofobicity and pharmacokinetics of the ^{99m}Tc-labeled small peptides (Miranda-Olvera et al., 2007).

We have reported a truncated peptide bombesin$_{(7-14)}$ which was conjugated with a bifunctional coupling agent HYNIC linked by β-Ala as a spacer. This analog was radiolabeled with technetium-99m, in the presence of the co-ligands tricine and EDDA, and showed suitable characteristics for tumor identification (de Barros et al., 2010b). However, this procedure presents some disadvantages, such as lengthy labeling procedures, high radiation exposure, and a greater risk of human error. The optimization of the labeling process is essential for the clinical use of radiopharmaceuticals. Kit formulation constitutes an alternative way to reach this goal, due to the fact that it can simplify the radiolabeling procedure and reduce radiation exposure during preparation. In addition, a kit formulation suitable for routine preparation of a radiotracer should be stable (long shelf-life) and yield high radiochemical purity at low cost (Smyth et al., 2005; Lee et al., 2007; Liu et al., 2009).

Therefore, the aim of this work was to develop a freeze-dried kit for labeling HYNIC-βAla-Bombesin$_{(7-14)}$ with technetium-99m, and evaluate its storage stability. Biodistribution studies and scintigraphic images were also performed in healthy mice to confirm long-term stability.

2. Materials and methods

2.1. Materials

The peptide HYNIC-βAla-Bombesin$_{(7-14)}$ was purchased from GL Biochem (Shanghai, China). Technetium-99m was obtained from an alumina-based ^{99}Mo/^{99m}Tc generator. All solvents and other reagents, including tricine, ethylenediamine-*N*,*N*′-diacetic acid (EDDA), and $SnCl_2 \cdot 2H_2O$, were purchased from Sigma-Aldrich (São Paulo, Brazil). All animal studies were approved by the local Ethics Committee for Animal Experiments (CETEA).

2.2. Kit formulation and radiolabeling

To an amber-colored vial containing 20 mg tricine and 5 mg of EDDA was added 0.5 ml of NaCl 0.9% (w/v). Next, 10 μg of HYNIC-βAla-Bombesin$_{(7-14)}$, and different amounts of $SnCl_2 \cdot 2H_2O$ (10–100 μg) were added. The pH was adjusted to 7. The vials were then frozen in boiling nitrogen and placed on the plate of a ModulyoD freeze-dryer (Thermo Electron Corporation, USA) at room temperature and a vacuum level of 10^{-1} atm. The condenser temperature was maintained at −45 °C. The drying process took 24 h. After this period, the freeze-dried bombesin kits with different amounts of $SnCl_2 \cdot 2H_2O$ ($KBBN_{10}$, $KBBN_{20}$, $KBBN_{40}$, $KBBN_{50}$, $KBBN_{100}$) were reconstituted with a fresh solution of $Na^{99m}TcO_4$ (37 MBq). The mixture was heated for 15 min in a water bath at 100 °C and then cooled in water. The radiochemical purity of such formulations was determined in order to establish the best $SnCl_2 \cdot 2H_2O$ concentration.

2.3. Radiochemical purity

The radiochemical purity was determined by Instant Thin Layer Chromatograph (ITLC) using a two solvent system: methylethylketone (MEK) to determine $^{99m}TcO_4^-$, and acetonitrile/water (1:1) to determine $^{99m}TcO_2$, as published elsewhere (de Barros et al., 2010b).

The HPLC analysis was performed using a Waters 717 with a radioactivity detector. HPLC solvents consisted of H_2O, containing 0.1% trifluoroacetic acid (solvent A) and acetonitrile containing 0.1% trifluoroacetic acid (solvent B). A Symmetry C-18 column (5.0 μm, 4.6 mm × 150 mm) was used at a rate of 1.0 ml/min. The HPLC gradient system began with a solvent composition of 95% A and 5% B, and followed a linear gradient of 30% A and 70% B for 10 min and 5% A: 95% B from 10 to 15 min.

2.4. Storage stability

Storage stability was investigated by using freeze-dried $KBBN_{50}$ ($n=3$). The lyophilization process was carried out as previously described. After freeze-dried during 24 h, $KBBN_{50}$ were stored in vacuum-sealed vials at −20 °C. At different time intervals (30, 60, 90, 120, and 180 day), freeze-dried $KBBN_{50}$ was taken and rehydrated with a fresh solution of $Na^{99m}TcO_4$ (37 MBq). The mixture was heated for 15 min in a water bath at 100 °C, and cooled in water. Afterwards, radiochemical purity was evaluated by ITLC and HPLC, as previously described.

2.5. In vitro stability

In vitro studies were performed for $KBBN_{50}$ after 180 days of storage. Stability was tested in NaCl 0.9% (w/v) and in mice plasma. The NaCl 0.9% (w/v) stability was evaluated at room temperature. Aliquots were taken out at 1, 2, 4, 6, and 24 h post-reconstitution, and analyzed by ITLC. Plasma stability was carried out in water bath at 37 °C under agitation. Similarly, aliquots were collected at the same time points and ITLC were performed.

2.6. Cell binding, internalization assay, and non-specific binding

Ehrlich tumor cells supplied in DMEM medium were diluted to 1×10^6 cells/tube and incubated with ^{99m}Tc-HYNIC-βAla-Bombesina$_{(7-14)}$ (0.3 nmol total peptide) in triplicate at 37 °C for 1, 4 and 8 h. The tubes were centrifuged (10 min, 3000*g*) and washed with 0.9% NaCl. The activity of the cell pellet was determined in an automatic scintillation apparatus. Radioactivity in the cell pellet represents both externalized peptide and internalized peptide. An aliquot with the initial activity was taken as 100%, and the cell uptake activity was then calculated.

To determine the percentage of internalization, the cell surface-bound radioligand (externalized peptide) was removed using an acid wash buffer (1 ml of 0.2 M acetic acid/0.5 M NaCl; pH 2.8) at room temperature for 5 min. The test tubes were centrifuged, washed with 0.9% NaCl, and re-centrifuged. Pellet activity represented internalization. Non-specific binding was performed in parallel using the same aforementioned protocol; however, 10 μM of the cold bombesin were used to block GRP receptors.

2.7. Biodistribution studies

Aliquots of the HYNIC-βAla-Bombesin$_{(7-14)}$ from $KBBN_{50}$, after 180 days of storage, were radiolabeled with 37 MBq of technetium-99m, then an aliquot of 3.7 MBq (~1 nmol) was injected intravenously into healthy Swiss mice ($n=5$). After 1, 4, and 8 h, mice were anesthetized with a mixture of xylazine (7.5 mg/kg) and ketamine (80 mg/kg) and then, sacrificed. Whole liver, spleen, kidneys, stomach, heart, lungs, blood, muscle, thyroid, and pancreas were removed, washed with distilled water, dried on filter paper, and placed in pre-weighed plastic test tubes. The radioactivity was measured by an automatic scintillation apparatus (Perkin Elmer, USA). A standard dosage containing the same injected amount was counted simultaneously in a separate tube, which was defined as 100% radioactivity. The results were expressed as the percentage of injected dose/g of tissue (%ID/g). Receptor blocking studies were also carried out by the administration