

Preparation and biodistribution study of ^{67}Ga -gallium calcitonin

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ABSTRACT

Introduction: In order to develop a radiolabeled calcitonin (CT) derivative for receptor imaging studies, CT was successively labeled with ^{67}Ga -gallium chloride.

Methods: The best results of the conjugation were obtained by the addition of 0.5 ml of a CT nasal pharmaceutical solution (1100 IU) to a glass tube pre-coated with DTPA-dianhydride (0.01 mg) at 25°C with continuous mild stirring for 30 min.

Results: After solid phase purification of the radiolabeled hormone, instant thin layer chromatography (ITLC) showed radiochemical purity of higher than 95 % at optimized conditions (specific activity =67-134 KBq/IU, labeling efficiency 70%). ^{67}Ga -DTPA-CT mainly accumulates in the liver.

Conclusion: Preliminary *in vivo* studies (ID/g%) in male wild-type rats showed significant liver uptake of the tracer after 24 hours. ^{67}Ga -DTPA-CT can be a suitable probe for biodistribution study of CT receptors in various physiological as well as neoplastic lesions with over-expressed calcitonin receptors.

Keywords: Calcitonin, Radiolabeling, Biodistribution, Ga-67

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INTRODUCTION

Calcitonin (CT) is a 32-amino acid linear polypeptide hormone with the molecular weight of 3454.93 D (1). It is produced in humans primarily by C-cells of the thyroid (2) which acts to reduce blood calcium, opposing the effects of parathyroid hormone (3) and is found in many other animals. The calcitonin receptor (CTR), found primarily on osteoclasts, is a G protein-coupled receptor (4). Calcitonin acts also directly on attenuation of subchondral bone turnover, and directly on chondrocytes, attenuating cartilage degradation and stimulating cartilage formation (5). Pharmaceutical CT is usually salmon calcitonin which is rapidly absorbed and eliminated. Peak plasma concentrations are attained within the first hour of administration. Animal studies have shown that calcitonin is primarily metabolized via proteolysis in human in the kidney following parenteral administration. The metabolites lack the specific biological activity of calcitonin. Bioavailability following subcutaneous and intramuscular injection in humans is high and similar for the two routes of administration (71% and 66%, respectively) (6).

Expression of CT and its receptor (CTR) is increased in advanced prostate cancer, and activated CT-CTR autocrine axis plays a pivotal role in tumorigenicity and metastatic potential of multiple prostate cancer cell lines. Recent studies suggest that CT promotes prostate cancer metastasis by reducing cell-cell adhesion through the disassembly of tight and adherens junctions and activation of beta-catenin signaling (7).

Many radiolabeled CT compounds have been developed for pharmacological studies. In one study, in vivo radiopharmacology of ^{68}Ga -labeled modified human CT was evaluated in rats using small animal PET and the arterial blood at different time points and urine were analyzed for radio-metabolites (8). For pharmacological

studies, ^3H -calcitonin was prepared and used for CTR biological activities be useful in exploring receptor interactions within the calcitonin peptide family (9) for drug development. Binding studies for salmon CT were also performed on rat membranes prepared from pons and medulla oblongata of rats using ^{125}I -CT compound (10). ^{67}Ga -labeled CT was prepared for preliminary biodistribution studies, based on our recent experiences on the preparation of radiometal-labeled proteins (11, 12). In this manuscript by developing a radiolabeled calcitonin complex a possible in vivo receptor study probe for calcitonin receptors has been developed.

METHODS

Production of ^{67}Ga was performed at the Agricultural, Medical and Industrial Research School (AMIRS, Karaj, Iran), 30 MeV cyclotron (Cyclone-30, IBA, Belgium). Enriched zinc-68 chloride with enrichment of >95% was obtained from Ion Beam Separation Department at AMIRS. All chemicals were purchased from commercial sources. Cyclic DTPA dianhydride was freshly prepared and kept under a blanket of N_2 . Miacalcin Injection (salmon calcitonin, 1500 IU) was a pharmaceutical sample purchased from Novartis Pharma Stein, Switzerland and was used without further purification. Instant thin layer chromatography (ITLC) was performed by counting Whatman No. 2 papers using a thin layer chromatography scanner, Bioscan AR2000, Bioscan Europe Ltd. (France). Analytical HPLC to determine the specific activity was performed by a Shimadzu LC-10AT, armed with two detector systems, flow scintillation analyzer (Packard-150 TR, U.S.A.) and UV-visible (Shimadzu, Japan) using Whatman Partisphere C-18 column 250 x 4.6 mm, Whatman, NJ, USA. Calculations were based on the 184 keV peak for ^{67}Ga . All values were expressed as mean \pm standard

deviation (Mean \pm SD) and the data were compared using Student T-test. Statistical significance was defined as $P < 0.05$. Animal studies were performed in accordance with the United Kingdom Biological Council's Guidelines on the Use of Living Animals in Scientific Investigations, 2nd edn. 1987. The approval of AMIRS ethical Committee was obtained for conducting this research. The percentages of injected doses in tissues (ID/g%) were determined using a high purity germanium (HPGe) detector coupled with a CanberraTM (model GC1020-7500SL) multichannel analyzer based on the area under the curve for 184 keV photopeak and calculated efficiency of the counting system.

Conjugation of cyclic DTPA di-anhydride with human recombinant CT

The chelator diethylenetriamine penta-acetic acid dianhydride was conjugated to the CT using a small modification of the well-known cyclic anhydride method (13). Conjugation was performed at a 1:1 molar ratio. In brief, 20 μl of a 1 mg ml^{-1} suspension of DTPA anhydride in dry chloroform (Merck, Darmstadt, Germany) was pipetted under ultrasonication and transferred to a glass tube. The chloroform was evaporated under a gentle stream of nitrogen. Commercially available CT (1500 IU, 1 ml, pH 8, \approx 3 nM) (14) was subsequently added and gently mixed at room temperature for 60 min.

Radiolabeling of CT conjugate with ^{67}Ga

The CT conjugate was labeled using an optimization protocol according to literature (15). Typically, 74 MBq of ^{67}Ga -chloride (in 0.2M HCl) was added to a conical vial and dried under a flow of nitrogen. To the ^{67}Ga containing vial, conjugated fraction was added in 1 ml of phosphate buffer (0.1 M, pH= 8) and mixed gently for 30 seconds. The resulting solution was incubated at room temperature for 30 minutes. Following incubation, the radiolabeled CT conjugate

was checked using ITLC/RTLC methods for the purity. In case of significant presence of impurities the sample can be purified using solid phase extraction using C_{18} Sep-Pak. Briefly, the column was pretreated with absolute ethanol (3 ml) and water (2 ml) respectively followed by the injection of radiolabeling mixture. The column was left at room temperature for 5 min and then was washed with water fractions (1 ml) till the flow-through activity in each fraction was less than 10 microcuries. Finally the radiolabeled compound was eluted from the column using 1ml-fractions of citrate buffer (pH. 5.5). Control labeling experiments were also performed using $^{67}\text{GaCl}_3$, and DTPA with $^{67}\text{GaCl}_3$.

Quality control of ^{67}Ga -CT

Paper chromatography: A 5 μl sample of the final fraction was spotted on a chromatography paper (Whatman No. 1. Whatman, Maidstone, UK), and developed in a mixture of 1mM DTPA in DDH_2O as the mobile phase.

Biodistribution of ^{67}Ga -DTPA-CT in wild-type rats

To determine its biodistribution, ^{67}Ga -DTPA-CT was administered to normal rats. A volume (50 μl) of final ^{67}Ga -DTPA-CT solution containing 40 ± 2 μCi radioactivity was injected intravenously to rats through their tail vein. The animals were sacrificed at exact time intervals (0.25, 0.5, 1, 2, 3 and 24h), and the specific activity of different organs was calculated as percentage of urea under the curve of 184 keV peak per gram using an HPGe detector.

RESULTS AND DISCUSSION

Salmon calcitonin is primarily and almost exclusively degraded in the kidneys, forming pharmacologically-inactive fragments of the molecule. Therefore, the

metabolic clearance is much lower in patients with end-stage renal failure than in healthy subjects. However, the clinical relevance of this finding is not known. Plasma protein binding is 30% to 40%. The metabolism of calcitonin-salmon has not yet been studied clinically.

Information from animal studies with calcitonin-salmon and from clinical studies with calcitonins of porcine and human origin suggest that calcitonin-salmon is rapidly metabolized by conversion to smaller inactive fragments, primarily in the kidneys, but also in the blood and peripheral tissues. A small amount of unchanged hormone and its inactive metabolites are excreted in the urine.

It appears that calcitonin-salmon cannot cross the placental barrier and its passage to the cerebrospinal fluid or to breast milk has not been determined. These results propose that developing a radiolabeled CT compound can be accomplished if the scanning and receptor binding studies would be performed within the first hour post injection using a short half life PET radionuclide such as ^{68}Ga , ^{18}F can be of great value.

In this study, we used ^{67}Ga radionuclide as a prototype for final ^{68}Ga radiolabeling and the optimization was performed using this nuclide for ultimate use. The labeling yield of ^{67}Ga -DTPA-CT has been studied in the wide range of CT/DTPA ratios in order to optimize the process and to improve ^{67}Ga -DTPA-CT performance in vitro. The overall radiolabeling efficiency was over 80%. Because of its isoelectric point (IEP), CT is soluble at pH 5.5-7 (16).

Figure 1, demonstrates the peptide sequence for salmon CT. Considering the existence of at least two lysine moieties in position 11 and 18, the NH_2 mediated conjugation through ccDTPA acylation looked feasible. Due to the existence of Cys_1 - Cys_7 disulfide bond the radiolabeling of the hormone in the presence of reductive materials such $^{99\text{m}}\text{Tc}$ can be problematic. The disulfide bond

increases the biological half life of the hormone in contrast with human analog.

The eluted fractions were checked for presence of radioactivity in order to determine the ^{67}Ga -DTPA-CT containing fractions. The fraction with the maximum radioactivity was chosen as the suitable final product for quality control and with appropriate specific activity for animal tests. The radiolabeling reached to 95% after 60 min.



Figure 1. Peptide sequence of the salmon calcitonin

In ITLC tests a 1-10 mM DTPA pH.4 solution was used as eluent in this system any free Ga cations would be complexed by the DTPA and the final complex would elute faster over the paper resulting in a peak with higher Rf.

While any remaining activity in the case of starting Ga used in the radiolabeling such as hydrolyzed and/or colloidal Ga would remain at the beginning (Rf. 0.1).

When radiolabeled with Ga, the Ga-DTPA-CT complex would remain at the beginning of the paper. Since the size and charges of the polypeptidic hormone are the major causes of attachment to the stationary phase a 2-4% radiochemical purity overestimation of the radiolabeled hormone could be considered due to the retention of hydrolysed Ga at the same Rf (0.04-0.1) (**Figure 2**).

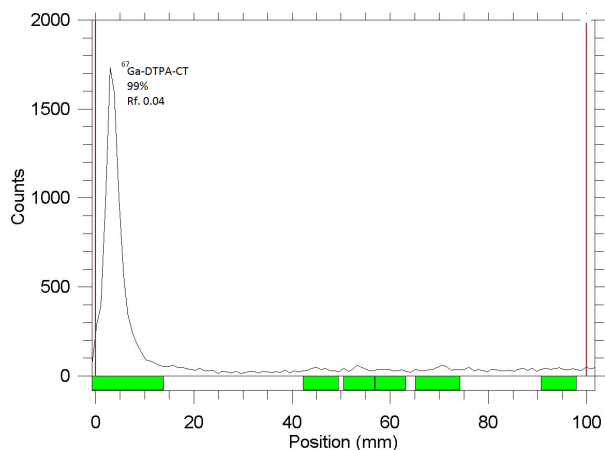


Figure 2. ITLC of final ^{67}Ga -DTPA-CT solution on Whatman paper using 10 mM DTPA solution as eluent.

At this stage the buffer eluted fraction with the highest activity was tested by HPLC in order to determine the radiochemical purity before administration to wild-type rats for biodistribution studies. [Figure 3](#) shows the HPLC chromatogram of $^{67}\text{Ga}^{3+}$. The fast eluting component (2.79 min) was shown to be a mixture of free ^{67}Ga which was washed out on reverse phase stationary phase. The radiolabeled protein was washed out at 14.78 minutes ([Figure 3](#)).

The stability of the radiolabeled protein *in vitro* was determined after challenge with phosphate-buffered saline and serum. ITLC analysis showed that the proteins retained the radiolabel over a period of 1 hour in final solution.

These results were confirmed by gel filtration chromatography. After incubation of ^{67}Ga -DTPA-CT with PBS for 2 h, almost the entire radioactivity eluted in the same position as ^{67}Ga -DTPA-CT; there was no evidence for large-scale release of free Ga. Similarly, gel filtration chromatography of ^{67}Ga -DTPA-CT after a 2 h incubation with human serum showed that the radioactivity still eluted in the same position. Thus, there was no evidence for either degradation or transchelation of ^{67}Ga to other serum proteins over a time period.

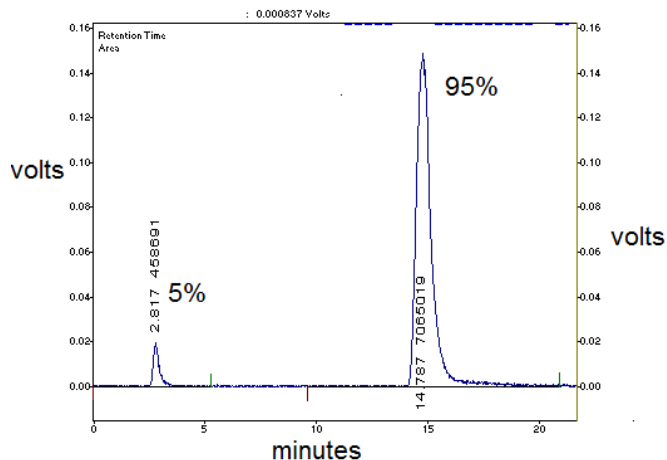


Figure 3. HPLC chromatogram of final radiolabeled solution on a reversed phase column using a gradient of acetate/citrate buffer.

The distribution of free $^{67}\text{GaCl}_3$ in appropriate buffer and ^{67}Ga -DTPA-CT among tissues were determined in male wild-type rats. A volume (0.1 ml) of final ^{67}Ga -DTPA-CT solution containing 40 μCi of radioactivity was injected into the dorsal tail vein. The total amount of radioactivity injected into each rat was measured by counting the 1-ml syringe before and after injection in a dose calibrator with a fixed geometry.

The animals were sacrificed by ether asphyxiation at selected times after injection (0.25h to 24 h), the tissues (blood, heart, spleen, kidneys, liver, intestine, muscle, bone, intestine, brain, stomach, lung, skin, pancreas, bladder and testes) were weighed and their specific activities were determined with a γ -ray scintillation as a percent of area under the curve of 184 keV per gram of tissue. For better comparison the biodistribution of free $^{67}\text{GaCl}_3$ was also determined ([Figures 4, 5](#)).

The kidneys account for approximately two-thirds of the metabolism of calcitonin, but relatively little is known regarding the details (17). Although in the radiolabeled form this might not be observed because gallium-67 is detached from the broken down peptides and no radioactivity would

not show up in the kidneys and urinary tract but majorly in liver as observed for gallium bioisoester, ferric cation.

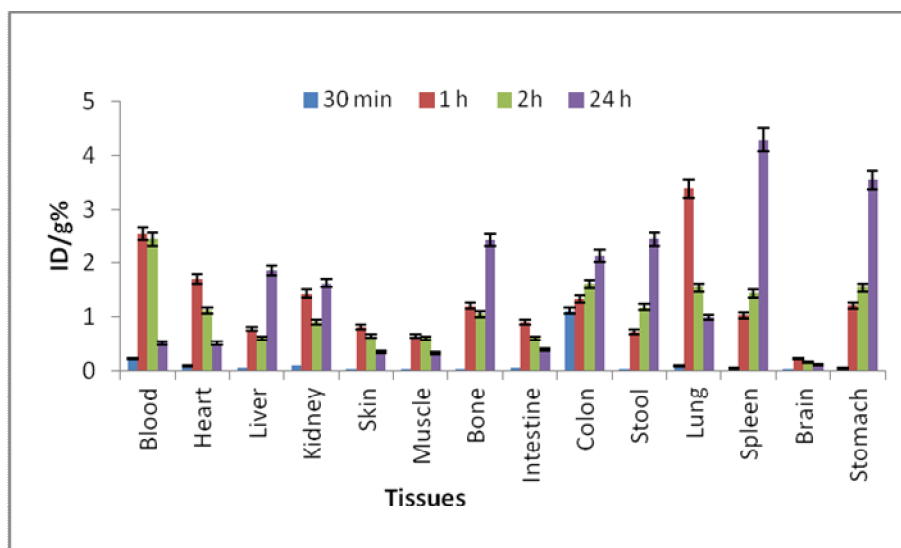


Figure 4. Biodistribution of $^{67}\text{GaCl}_3$ (40 μCi) in wild-type rats 0.5-24h after iv injection via tail vein (ID/g%: percentage of injected dose per gram of tissue calculated based on the area under curve of 184 keV peak in gamma spectrum).

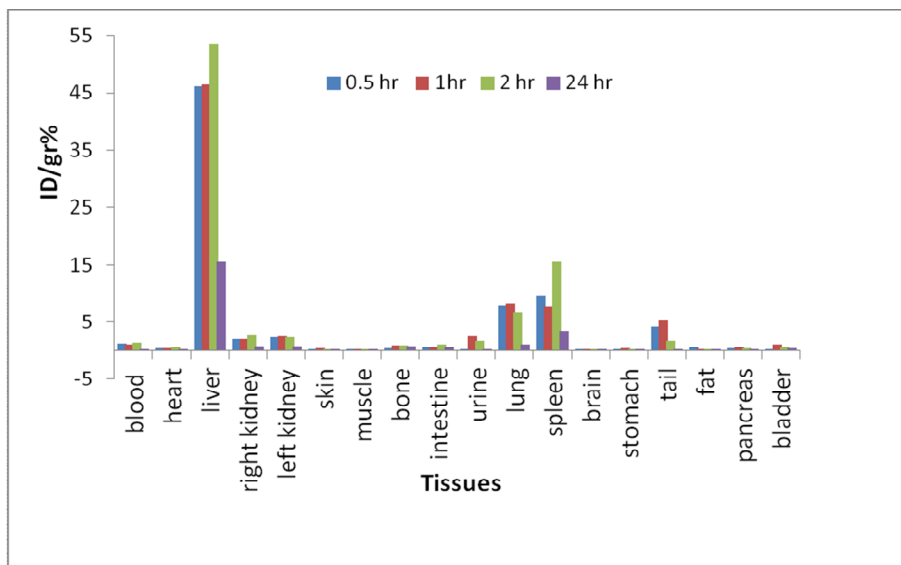


Figure 5. Biodistribution of $^{67}\text{Ga-DTPA-CT}$ (1.85 MBq, 40 μCi) in wild-type rats 0.5-24h after iv injection via tail vein (ID/gr%: percentage of injected dose per gram of tissue calculated based on the area under curve of 184 keV peak in gamma spectrum).

Like many other radiolabeled peptides and proteins ^{67}Ga -DTPA-CT mainly accumulates into liver, while lung and spleen also show less accumulation mostly due to the release of the Ga from the labeled compound in the time limit following injection, this may also be caused by the fact that the hormone itself brakes down into smaller peptides as a part of biodegradation.

The radiolabeled hormone is rapidly removed from the circulation and a possible low serum protein binding exists for this hormone. Skin, muscle, brain, pancreas and also kidney did not demonstrate remarkable uptakes however tracer activity in spleen was significant.

CONCLUSION

Total labeling and formulation of ^{67}Ga -DTPA-CT took about 60 minutes. A suitable specific activity product was formed *via* insertion of ^{67}Ga cation. No other labeled conjugates were observed upon RTLC and/or HPLC analysis of the final preparations. The radio-labeled complex was stable in human serum for at least 2 hours and no significant amount of free ^{67}Ga as well as ^{67}Ga -DTPA was observed. A radiochemical purity of 95% was detected by HPLC. The final preparation was administered to wild-type rats and biodistribution of the radiopharmaceutical was checked 0.5 to 24 hours later. The radiolabeled hormone is cleared from blood circulation and most of the tracer accumulates in livers. After 24 h the major tracer uptake is observed in liver and also lungs and spleen. ^{67}Ga -DTPA-CT can be a suitable probe for biodistribution study of CTR receptors in various physiological and neoplastic diseases with over-expressed CTRs.

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