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Development of a radiolabeled glucagon compound for imaging

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Abstract. In order to develop a possible Ga-labeled glucagon (GCG) compound for imaging studies, biosynthetic glucagon (GCG) was labeled with [^{67}Ga]-gallium chloride after conjugation with freshly prepared diethylenetriaminepentaacetic acid dianhydride (ccDTPA). After solid phase purification of the radiolabeled hormone, high performance liquid chromatography (HPLC) and instant thin-layer chromatography (ITLC) showed a radiochemical purity around 95% in optimized conditions (specific activity = 296–370 GBq/mM; labeling efficiency 85%). Preliminary *in vivo* studies (ID·g^{-1}%) in male wild-type rats showed heart:muscle, liver:muscle, lung:muscle and stomach:muscle ratios to be 5.53, 2.9, 7.56, 3.69, 3.2 (in 5 min), respectively while after 2 h liver:blood, lung:blood and spleen:blood ratios were 14.21, 16.86 and 7.8, respectively. The data suggests 5 min post injection, the tracer is accumulated in GCGR rich tissues which is in agreement with biodistribution studies and reported GCG receptors (GCGRs). The results of the present study can possibly offer a candidate for non-invasive imaging of glucagon receptor related diseased and malignancies such as glucagonoma.

Key words: glucagons • radiolabeling • biodistribution • Ga-67

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Introduction

Glucagon (GCG) is a linear peptide of 29 amino acids. Its primary sequence is almost perfectly conserved among vertebrates. GCG helps to maintain the level of glucose in the blood by binding to glucagon receptors (GCGRs) on hepatocytes, causing the liver to release glucose, stored in the form of glycogen, through a process known as glucogenolysis.

^{125}I-GCG is the only radiolabeled GCG compound that has been reported in the literature according to our knowledge and is frequently used in radio-pharmacological studies. ^{125}I-GCG has been used in the study of GCG hydrolysis by proximal tubules, identification of renal extraction mechanisms [20], GCG receptor binding [7], rat brain binding [9], reabsorption measurements in urinary tract [4] and hormone internalization in hepatocyte [3]. Also the photoreactive ^{125}I-GCG was used to label GCGRs [6].

The presence of GCGRs in various human malignancies has been well documented. For instance, glucagonoma is a neuroendocrine tumour that develops from glucagon-producing pancreatic cells. They are usually slow-growing, but generally advanced at diagnosis, and metastatic disease is virtually incurable. Liver is the most common site of metastatic disease [19].

The diagnosis of pancreatic endocrine tumors, such as glucagonomas, is difficult and requires a careful history and examination combined with laboratory tests

and radiologic imaging. Surgical resection remains the treatment of choice even in the face of metastatic disease. Further development of novel diagnostic and treatment modalities offers potential to greatly improve quality of life and prolong disease-free survival for patients with pancreatic endocrine tumors [5]. Due to overexpression of GCGRs on these malignant cells, the development of an appropriate radiolabeled compound capable of nuclear medicine imaging using single photon emission computed tomography (SPECT) and positron emission tomography (PET) can be of great importance. According to our knowledge, there were no reports of radiolabeled GCG for imaging studies in the literature. In this work, following the preparation of a GCG conjugate for the use in diagnostic GCGR studies. ^{67}Ga-GCG was prepared and used for preliminary biodistribution studies, based on our recent experiences on the preparation of radiometal-labeled proteins [13].

Experimental

Materials

Production of ^{67}Ga was performed at the Agricultural, Medical and Industrial Research School (AMIRS, Karaj, Iran) using a 30 MeV cyclotron (Cyclone-30, IBA, Belgium). Enriched zinc-68 chloride (enrichment > 95%) was obtained from the Ion Beam Separation Department at AMIRS. All chemicals were purchased from commercial sources. GlucaGen® (glucagon [rDNA origin] for injection) manufactured by Novo Nordisk A/S (1 mg/ml, 1 IU/ml) and was used without further purification. Cyclic DTPA dianhydride was freshly prepared and kept under a blanket of N_2. 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 (Japan) instrument, armed with two detector systems, a flow scintillation analyzer (Packard-150 TR, USA) and a UV-visible (Shimadzu, Japan) using Whatman Partisphere C-18 column (250 × 4.6 mm), Whatman, USA. Solid phase purification of the radiolabeled hormone was performed using C_{18} Sep-Pak from Waters Co. (USA). Calculations were based on the 184 keV peak for ^{67}Ga. All values were expressed as mean ± standard deviation and the data were compared using Student's t-test. Animal studies were performed in accordance with the United Kingdom Biological Council's Guidelines on the Use of Living Animals in Scientific Investigations, 1987. The approval of AMIRS Ethical Committee was obtained for conducting this research. The wild-type rats (NMRI) were purchased from the Pasteur Institute of Iran, Karaj, all weighing 180–200 g; they were acclimatized at a proper rodent diet and 12 h/12 h day/night light/darkness. The percentage of injected dose in tissue (ID·g^{-1}%) were determined using a high-purity germanium (HPGe) detector coupled with a Canberra™ (model GC1020-7500SL, USA) multichannel analyzer based on the area under the curve for 184 keV photopeak and calculated efficiency of the counting system.

Procedures

Production of ^{67}Ga

^{68}Zn(p,2n)^{67}Ga was used as the best nuclear reaction for the production of ^{67}Ga. Impurities could be removed in a radiochemical separation process. After the target bombardment process, chemical separation was carried out in no-carrier-added form. The irradiated target was dissolved in 10 mol·L^{-1} HCl (15 ml) and the solution was passed through a cation exchange resin (AG 50W, H^+ form, mesh 200–400, h: 10 cm, Ø: 1.3 cm) which had been preconditioned by passing 25 mL of 9 mol·L^{-1} HCl. The column was then washed by 25 mL of 9 mol·L^{-1} HCl at a rate of 1 mL/min to remove copper and zinc ions. To the eluent 30 mL of water plus about 100 mL of a 6 mol·L^{-1} HCl solution was added. The latter solution was loaded on another exchange resin (AG1X8 Cl^- form, 100–200 mesh, h: 25 cm, Ø: 1.7 cm) pretreated with 6 mol·L^{-1} HCl (100 mL). Finally, the gallium-67 was eluted as [^{67}Ga]$GaCl_3$ using 2 mol·L^{-1} HCl (50 mL); the whole process took about 60 min.

Radionuclide purity control

Gamma spectroscopy of the final sample was carried out by counting the activity in a high-purity germanium (HPGe) detector coupled to a Canberra™ multichannel analyzer for 1000 s.

Chemical purity control

The presence of zinc and copper cations were checked by the polarography method. The area under curve of polarogram of the test samples were lower than the standards even at 1 ppm of standard zinc and copper [15].

Conjugation of ccDTPA with human recombinant hCG

The chelator ccDTPA was conjugated to the GCG using a small modification of the well-known cyclic anhydride method [8]. 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, Germany) was pipetted under ultrasonication and transferred to a glass tube. The chloroform was evaporated under a gentle stream of nitrogen. Commercially available GCG (1 mg, 1 mL, pH 6, ≈ 0.3 nmol^{-1}) was subsequently added and gently mixed at room temperature for 60 min followed by size exclusion chromatography.

Radiolabeling of GCG conjugate with ^{67}Ga

The GCG conjugate was labeled using an optimized protocol according to the literature [14]. Typically, 74 MBq of ^{67}Ga-chloride (in 0.2 mol·L^{-1} HCl) was added to a conical vial and dried under a flow of nitrogen. To the ^{67}Ga containing vial, the conjugated fraction was added in 1 mL of phosphate buffer (0.1 mol·L^{-1}, pH 6) and mixed gently for 30 s. The resulting solution was incubated at room temperature for 30 min. Following incubation, the radiolabeled GCG conjugate was checked using for purity the ITLC/RTLC methods. In the case of presence of unreacted amounts of im-

purities, 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 μCi. Finally, the radiolabeled compound was eluted from the column using 1 mL fractions of citrate buffer (pH 5.5). Control labeling experiments were also performed using $^{67}GaCl_3$, and DTPA with $^{67}GaCl_3$.

Quality control of [^{67}Ga]-GCG

Paper chromatography. A 5-μL sample of the final fraction was spotted on a chromatography paper (Whatman no. 2, Whatman, UK), and developed in a mixture of 1 mol·L^{-1} DTPA in H_2O as the mobile phase.

High performance liquid chromatography. HPLC was performed on the final preparation using acetate buffer solution (50 Mmol·L^{-1} pH 5.5) as eluent A (flow rate: 1 ml/min) for 20 min in order to elute low molecular mass components. Radiolabeled peptide was eluted using a gradient of the latter solution (100 to 0%) and citrate buffer solution B (50 mM, pH 4,0 to 100%, 5 min A;100%, B;0%, 5 min A;70%, B;30, 5 min A;50%, B;50%, 50 A:0%, B;100%) using reverse stationary phase.

Stability testing of the radiolabeled compound

Stability of ^{67}Ga-DTPA-GCG in phosphate buffer solution was determined by storing the final solution at 4°C for 4 h and performing frequent ITLC analysis to determine radiochemical purity. ITLC analysis of the conjugated product was also performed to monitor degradation products or other impurities after the conjugated DTPA-GCG was stored at –20°C for more than 1 month. After subsequent ^{67}Ga-labeling of the stored conjugated product, both labeling efficiency and radiochemical purity were determined.

Stability testing of the radiolabeled compound in presence of serum

Labeled compound stability in serum, was assessed by gel filtration on a Sepharose column (1 × 30 cm). The column was equilibrated with PBS and eluted at a flow rate of 0.5 mL·min^{-1} at room temperature; 0.5 mL fractions were collected.

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

To determine its biodistribution, ^{67}Ga-DTPA-GCG was administered to wild-type rats. A volume (50 μl) of final ^{67}Ga-DTPA-GCG solution containing 40±2 μCi radioactivity was injected intravenously to rats through their tail vein.

The animals were sacrificed at exact time intervals (5, 15 min, 1, 2, 4, 24, 48 and 72 h). 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.

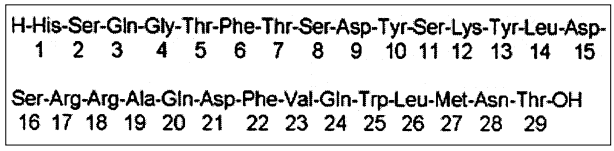

Fig. 1. Amino acid sequence of GCG.

Results

Glucagon with a molecular weight of 3483, is a single-chain polypeptide containing 29 amino acid residues (isoelectric point pI 7) is synthesized and secreted from A cells of pancreatic islets scattered throughout the islet. The liver and kidney seem to be the major sites of glucagon catabolism, but the relative contribution of each remains unclear.

In this work, the labeling yield of ^{67}Ga-DTPA-GCG has been studied in a wide range of GCG/DTPA ratios in order to optimize the process and to improve ^{67}Ga-DTPA-GCG performance *in vitro*. The overall radiolabeling efficiency was over 85%. Because of its isoelectric point (IEP) of around 7, GCG is soluble in lower physiological serum pH (5.5–6) being adequately stable hypothetically [18]. Figure 1 demonstrates the peptide sequence for GCG and considering the existence of one lysine moiety in the structure, the NH_2 mediated conjugation through ccDTPA acylation looked feasible, leading to a possible 1:1:1 stoichiometry of the DTPA:GCG:Ga ratio, which was a suitable condition.

The protein was conjugated using ccDTPA in a similar way already reported, followed by size exclusion chromatography of the compound showing 78–85% radiochemical purity after 1 h. Due to the relative instability of the radiolabeled peptide at room temperature instead of increasing time to obtain higher purities, solid-phase extraction using C_{18} column was used. The radiolabeled mixture was loaded on the preconditioned C_{18} Sep-Pak. Eluting the loaded column with water, removed free $^{67}Ga^{3+}$ as well as ^{67}GaDTPA due to their ionic properties. After purging the column with nitrogen for 5 min, the radiolabeled protein was eluted using citrate buffer in the first 3 elutions (1 mL).

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

At this stage, the buffer eluted fraction with the highest activity was tested by ITLC and HPLC in order to determine the radiochemical purity before administration to wild-type rats for biodistribution studies. Figure 2 shows the ITLC chromatograms for free $^{67}Ga^{3+}$ and the labeled compound after solid phase extraction.

Figure 3 demonstrated the HPLC chromatogram of $^{67}Ga^{3+}$ which was tested as a control. In HPLC experiments of the radiolabeled compound, two major peaks can be observed. The fast eluting component (2.79 min) was shown to be a mixture of free ^{67}Ga and ^{67}GaDTPA which was washed out on the reverse phase stationary phase. The radiolabeled protein was washed out at 20.01 min (Fig. 4).

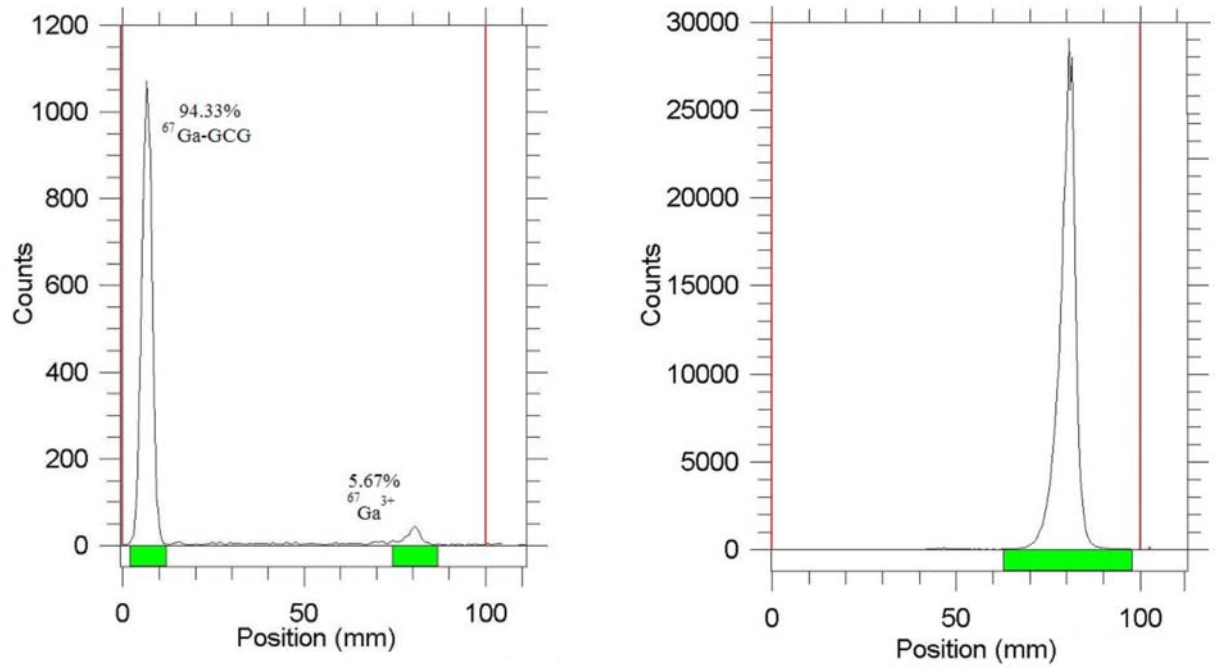

Fig. 2. ITLC of free ^{67}Ga used in the radiolabeling (right) and ^{67}Ga-GCG solution (left) on Whatman paper using 10 mM DTPA solution as eluent.

Considering the amount of activity used (74 MBq) and the radiochemical purity of the final purified sample (95%), a specific activity of 22–23 TBq·mmol^{-1} has been obtained after Sep-Pak purification.

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 h in the final solution.

These results were confirmed by gel filtration chromatography. After incubation of [^{67}Ga]-DTPA-GCG with PBS for 2 h, there was no change in the R_f for [^{67}Ga]-DTPA-GCG and also there was no evidence for a large-scale release of free Ga resulting in the appearance of any new radiopeak.

Gel filtration chromatography of [^{67}Ga]-DTPA-GCG after incubation for 2 h with human serum showed that 25% of the radioactivity is not eluted in the same fraction. Thus, there is a fraction of the tracer which has degraded or transchelated ^{67}Ga to other serum proteins over this time period. Also the biodistribution data supports this observation.

The distribution of free $^{67}GaCl_3$ in appropriate buffer has been already reported elsewhere [12]. Figure 5 demonstrates the biodistribution of [^{67}Ga]-DTPA-GCG among tissues in male wild-type rats.

A volume (0.1 ml) of final [^{67}Ga]-DTPA-GCG solution containing 40 μCi of radioactivity was injected into the rats' 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 CO_2 asphyxiation at selected times after injection (5 min–72 h), the tissues (blood, heart, spleen, kidneys, liver, intestine, muscle, bone, brain, stomach, lung, skin, fat, pancreas and bladder) were weighed and their specific activities were

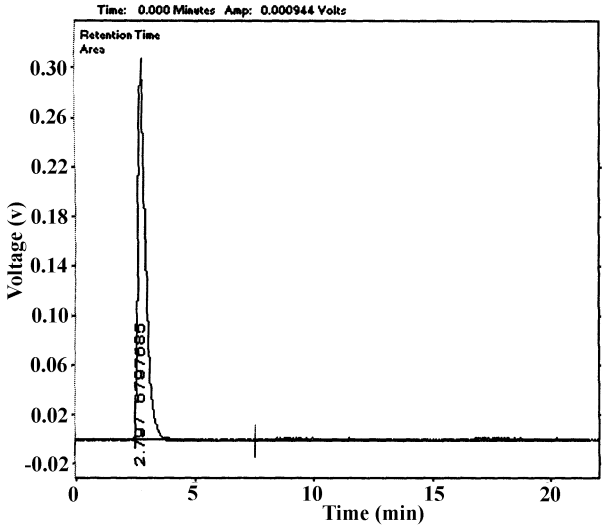

Fig. 3. HPLC chromatogram of free $^{67}GaCl_3$ solution on a reversed phase column using a gradient of acetate/citrate buffer.

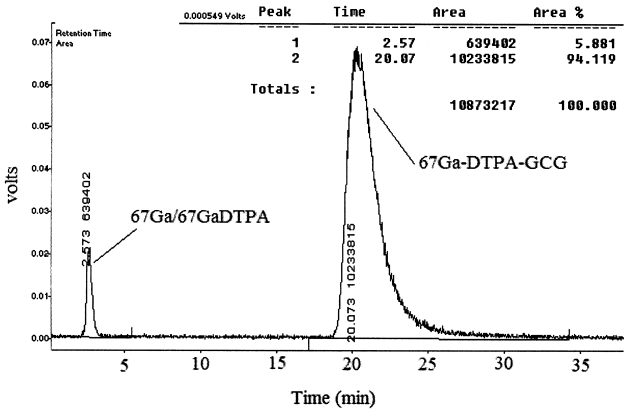

Fig. 4. HPLC chromatogram of SPE purified final radiolabeled solution on a reversed phase column using a gradient of acetate/citrate buffer.

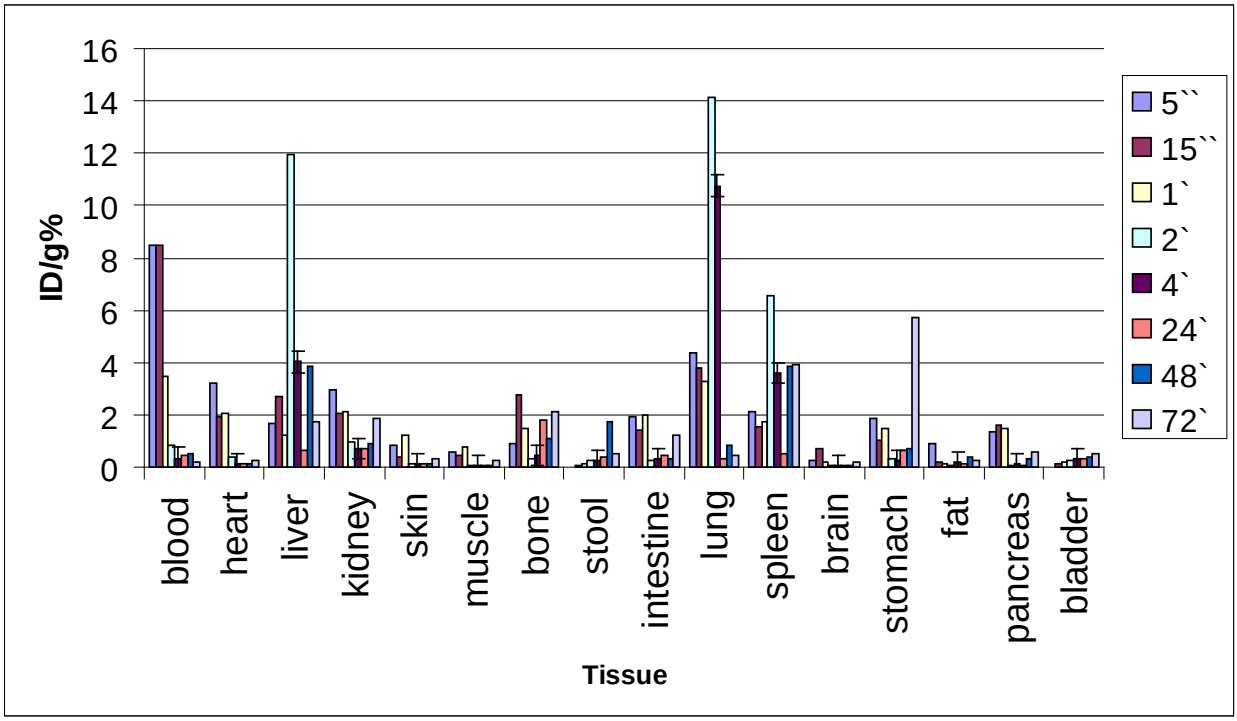

Fig. 5. Biodistribution of [^{67}Ga]-DTPA-GCG (1.85 MBq, 40 μCi) in wild-type rats 5 min–72 h after IV injection via tail vein (ID·g^{-1}%: percentage of injected dose per gram of tissue calculated based on the area under curve of 184 keV peak in gamma spectrum).

determined with a γ-ray scintillation detector as a percent of area under the curve of 184 keV per gram of tissue.

The tracer is removed from the blood stream after 1 h and this is in accordance with biodistribution pattern for most radiolabeled small proteins and peptides.

Glucagon receptors are mainly expressed in liver and in kidney with lesser amounts found in heart, adipose tissue, spleen, thymus, adrenal glands, pancreas, cerebral cortex, and gastrointestinal tract.

Heart uptake demonstrates a significant uptake after 5 min post injection (3–4%), but due to possible degradation as reported [2], the accumulation decreases after 60 min.

Liver is a high uptake organ possibly due to two different mechanisms; a) the presence of high GCGRs in the hepatocytes mediating the glycogenolysis and b) liver is repeatedly reported as the major uptake tissue for proteins and some other macromolecules, this bi-mechanistic pattern can also be supported by a significant decrease in liver uptake after 1 h. 5–15 min post injection, the uptake is increasing and this can be due to the direct receptor : ligand interaction, but after 1 h the 2 d increase is possibly caused by non-specific protein uptake in the liver. Degradation processing may occur locally in target tissues such as the pancreas, liver or heart, as well as in the circulation [14].

It has long been known that the kidney is capable of degrading glucagon. Arteriovenous gradients across the kidney in normal animals infused with glucagon indicate extraction of 23 to 39% of the presented glucagon [1, 19]. Because less than 2% of the extracted hormone appears in urine and because nonfiltering kidneys continue to extract appreciable amounts of glucagon, it seems that both tubular re-absorption and postglomerular capillary tubular uptake precede renal parenchymal degradation of glucagon. Parenteral administration of glucagon produces relaxation of the smooth muscle of the stomach, duodenum, small bowel and colon. This indirectly proposes the existence of GCGRs in GI tract, as it can be obviously observed in stomach 48–72 h post injection.

Five min post injection, the heart:muscle, liver : muscle, lung : muscle and stomach : muscle ratios were 5.53, 2.9, 7.56 and 3.69, respectively while after 2 h the liver : blood, lung : blood and spleen : blood ratios were 14.21, 16.86 and 7.8, respectively.

The half-life of glucagon in plasma is approximately 3 to 6 min [10], while under the circumstances in IV injections it has been reported to be 25–30 min [11], thus the main receptor binding takes place in the first 1–20 min post injection, although the accumulation at longer time intervals at the receptor rich tissues is also observed. This can be possibly caused by the unknown cell accumulation of the tracer and/or the metabolites. Also a slight rat brain uptake can be observed on 15 min post injection (1–2%) which is in accordance with previous reports [9]. Although glucomoma has been known for some time past, the diagnosis has not been well established yet. Arterial stimulation and venous sampling (ASVS) is known to be useful for insulinoma and gastrinoma, and just recently its usefulness for glucagonoma has been verified using this invasive method followed by sampling and tissue studies [17]. The results of the present study can possibly offer a candidate for non-invasive imaging of glucagon receptor related diseased and malignancies such as glucagonoma. Also the early diagnosis of this malignancy is a major breakthrough for the therapy, while it has just recently been reported that this malignancy can develop hepatic metastasis [16]. The results of the present study can possibly offer a candidate for non-invasive imaging of glucagon receptor related diseases and malignancies such as glucagonoma.

Although many radioiodine labeled glucagons are reported for *in vitro* studies, none of them contain I-123 compound, suitable for imaging while this compound can be a good candidate as well. Considering the biological half-life of native glucagon (25–30 min) and intermediate I-123 half-life (13.2 h), the radiolabeling of the GCG with radioiodine with I-123 does not seem appropriate, while Ga-68, a widely used PET radionuclide (half-life 68 min), seems an interesting candidate for developing a tracer. Thus, in this work the radiolabeling GCG performed using Ga-67 radionuclide due to availability in our center. The optimized method and conditions can easily be used for Ga-68 labeling.

Discussion

Total labeling and formulation of [^{67}Ga]-DTPA-GCG took about 60 min. A suitable specific activity product was formed via insertion of ^{67}Ga cation. No other labeled conjugates were observed upon ITLC and/or HPLC analysis of the final preparations. The radiolabeled complex was stable in human serum for at least 1 h 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 5 min to 72 h later. Preliminary *in vivo* studies (ID·g^{-1}%) in male wild-type rats showed a significant heart and liver uptake of the tracer after 5 min, in agreement with the biodistribution studies and reported GCG receptors (GCGRs). Tissue : muscle values extracted from tissue accumulated activities demonstrate that 5 min post injection the tracer is possibly accumulated in GCGR rich tissues. [^{67}Ga]-DTPA-GCG can be a suitable probe for biodistribution study of CGR in various physiological and malignant diseases with over-expressed CGRs. Due to interesting characteristics of ^{68}Ga radionuclide (half-life 68 min) in molecular imaging, and biological half-life of GCG, developing a ^{68}Ga-labeled tracer can be of great interest. The results of the present study can possibly offer a candidate for non-invasive imaging of glucagon receptor related diseased and malignancies such as glucagonoma.

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