NUKLEONIKA 2007;52(4):145–151

ORIGINAL PAPER

Preparation and biodistribution of [^{67}Ga]-insulin for SPECT purposes

Amir R. Jalilian, Javad Garousi, Ebrahim Gholami, Mehdi Akhlaghi, Fatemeh Bolourinovin, Saeed Rajabifar

Abstract. Human recombinant insulin was successively labeled with [^{67}Ga]-gallium chloride after conjugation with freshly prepared cyclic DTPA-dianhydride (ccDTPA). The best results of the conjugation were obtained by the addition of 0.5 ml of an insulin pharmaceutical solution (5 mg/ml, in phosphate buffer, pH = 8) to a glass tube precoated with DTPA-dianhydride (0.01 mg) at 25°C with continuous mild stirring for 30 min. Radiothin-layer chromatography (RTLC), instant thin-layer chromatography (ITLC) and high-performance liquid chromatography (HPLC) showed overall radiochemical purity higher than 96% in optimized conditions (specific activity = 300–500 MBq/mg, labeling efficiency 77%). Preliminary *in vivo* studies with normal rats were performed to determine the biodistribution of the radiotracer up to 110 h. They showed a high liver uptake of the tracer which is consistent with other reported radiolabeled insulins.

Key words: radiogallium • insulin • radiolabeling • biodistribution • radiopharmaceutical • cyclotron

A. R. Jalilian✉, J. Garousi, E. Gholami, M. Akhlaghi, F. Bolourinovin, S. Rajabifar
Nuclear Medicine Group,
Agriculture, Medicine and Industrial Research School (AMIRS-NSTRI),
P. O. Box 31485-498, Karaj, Iran,
E-mail: ajalilian@nrcam.org, arjalilian@yahoo.com

Received: 19 June 2007
Accepted: 30 July 2007

Introduction

Metabolism of glucose has been an interesting target for tumor imaging for 3 decades. Development of radiolabeled glucose by many research groups in the 1970s and 1980s led to development of the most common PET radiopharmaceutical, ^{18}F-FDG. Since then, ^{18}FDG has been used for the detection and staging of several malignancies. Few limitations have been proposed in the last few years in using FDG as a tracer, including natural accumulation of the tracer in the bladder, liver and brain which can limit the detection of malignancies of these regions.

It has already been shown insulin stimulates phosphorylation of its own receptor [17, 18] and also phosphorylation of the insulin receptor may be an early step in insulin action which happens in 80% of human cells with some exceptions such as neurons. The idea of detecting FDG consumption in cells indirectly by insulin radiolabeling has come to our interest. Insulin is a peptide pharmaceutical which consists of 51 amino acids which are oriented in two chains (α- and β-chain).

Insulin has been radiolabeled with many SPECT and PET radionuclides such as ^{99m}Tc [1], ^{123}I [11, 22] and ^{18}F [4, 21] for various purposes such as a study of insulin biodistribution in diabetic subjects [4] and overexpression of the insulin receptor on IM-9 lymphoblastoid tumor cell surface [10].

In order to obtain an insulin conjugate for use in diagnostic studies using metallic PET or SPECT radioisotopes, ^{67}Ga-labeled insulin was prepared for preliminary biodistribution studies, based on our recent

Table 1. Physical properties of two PET gallium radioisotopes

Properties	^{68}Ga	^{67}Ga	^{66}Ga
Gamma energies (keV)	511(β^+)	93.3 184.6 300.2	511(β^+) 834 1039 2752
Positron energy	1900(β^+)	–	4153(β^+)
Mode of decay	10% EC to ^{68}Zn 90% β^+	EC to ^{67}Zn	43% EC to ^{66}Zn 57% β^+
Half-life	68 min	78.3 h	9.5 h
Route of production	^{68}Ge daughter ^{66}Zn(α,2n)^{68}Ge	^{68}Ga(p,2n)^{67}Ga	^{66}Zn(p,n)^{66}Ga
Possible impurity	^{68}Ge	^{66}Ga, ^{65}Zn	^{65}Zn
Proton energy (MeV)	12–22	24	6–15

experiences on the preparation of radiometal-labeled proteins [13–15].

The positron-emitting Ga(III) radionuclides, ^{66}Ga and ^{68}Ga, have been proposed for the applications in positron emission tomography imaging (PET) [6, 19]. ^{66}Ga ($T_{1/2}$ = 9.49) is an intermediate-lived radionuclide that is potentially suitable for positron emission tomography imaging of biological processes with an intermediate to slow target tissue uptake [7, 8, 24] (Table 1).

We have recently reported the production of this radionuclide using the ^{66}Zn(p,n)^{66}Ga reaction [20] and now we are working on the production of ^{68}Ge/^{68}Ga generators for remote nuclear medicine centers. A precise labeling strategy was employed using a freshly-prepared DTPA cyclic dianhydride, with various insulin concentrations and using available gallium-67 for optimization of radiolabeling conditions due to a longer half-life in contrast to PET gallium radionuclides. Insulin is exceedingly sensitive to external influences. Such influences include heat and alkali sensitivity, sensitivity with respect to oxidizing and reducing agents, as well as to strong acidly reacting substances. Thus, radiolabeled insulins, using ^{99m}Tc and radioiodine, that mostly benefit from oxido/redox reactions always impose risk of biological activity loss. Finally, an optimized radiolabeling method for developing a highly reactive DTPA-conjugated insulin for possible diagnostic studies was introduced.

Experimental

Materials

Production of ^{67}Ga was performed at the Agriculture, Medicine and Industrial Research School (AMIRS), using a 30 MeV cyclotron (Cyclone-30, IBA). Enriched zinc-68 chloride (> 95%) was obtained from the Ion Beam Separation Department at NRCAM. Sephadex G-50, sodium acetate, phosphate buffer components methanol and ammonium acetate were purchased from Sigma-Aldrich Chemical Co. (UK). Cyclic DTPA dianhydride was freshly prepared and kept under a blanket of N_2. Insulin was a pharmaceutical sample purchased from Lorestan Pharmaceutical Co. (Borojerd, Iran) and was used without further purification. Radio-chromatography was performed by counting different 5 mm slices of polymer-backed silica gel paper using a high purity germanium (HPGe) detector coupled with a Canberra™ (model GC1020-7500SL) multichannel analyzer. Analytical HPLC to determine the specific activity was performed by a Shimadzu LC-10AT, armed with two detector systems, a flow scintillation analyzer (Packard-150 TR) and a UV-visible (Shimadzu) using a Whatman Partisphere C-18 column 250 × 4.6 mm, Whatman, NJ, USA. Calculations were based on the 184 keV peak for ^{67}Ga. All values were expressed as mean ± standard deviation (Mean ± SD) and the data were compared using the 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 ed.

Methods

Production of ^{67}Ga

The reaction ^{68}Zn(p,2n)^{67}Ga was used as the best nuclear reaction for the production of ^{67}Ga. An enriched zinc-68 layer (80 μm) electrodeposited on a copper support was irradiated by 24 MeV protons with a beam intensity of 160 ± 20 μA (integral 1400 μAh). The expected impurities are copper, Zn-68 and Zn-65. Impurities could be removed in the 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 M 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 M HCl. The column was then washed with 25 ml of 9 M 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 M

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 M HCl (100 ml). Finally, the gallium-67 was eluted as [^{67}Ga]$GaCl_3$ using 2 M HCl (50 ml); the whole process took about 60 min.

Control of radionuclide purity

Gamma spectroscopy of the final sample was carried out by counting the radioactivity with the HPGe detector coupled to the Canberra™ multichannel analyzer for 1000 s.

Chemical purity control

The presence of zinc and copper cations were checked by the polarography method. Even at 1 ppm of standard zinc and copper concentrations, the area under curve of polarogram of the test samples were lower than the standards.

Conjugation of cyclic DTPA dianhydride with human recombinant insulin

The chelator diethylenetriamine penta-acetic acid dianhydride was conjugated to the insulin using a small modification of the well-known cyclic anhydride method [12]. Conjugation was performed at a 1:1 molar ratio. In brief, 20 µl of a 1 $mg \cdot ml^{-1}$ 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 insulin (5 mg, 0.5 ml, pH 8) was subsequently added and gently mixed at room temperature for 60 min. The conjugation mixture was then passed through a Sephadex G-50 column (2 × 15 cm, 2 g in 50 ml of Milli-Q® water) separately and one-milliliter fractions were collected and checked for the presence of protein using UV absorbance at 280 nm or visible folin-phenol colorimetric assay. The fractions containing the highest concentration of the conjugate were chosen and kept at 4°C and for radiolabeling.

Radiolabeling of insulin conjugate with ^{67}Ga

The insulin conjugate was labeled using an optimization protocol according to the literature [5, 16]. Typically, 37–40 MBq of ^{67}Ga-chloride (in 0.2 M HCl) was added to a conical vial and dried under a flow of nitrogen. To the Ga containing vial, a conjugated fraction was added in 1 ml of phosphate buffer (0.1 M, pH = 8) and mixed gently for 30 s. The resulting solution was incubated at room temperature for 30 min. Following incubation, the radiolabeled insulin conjugate was checked, using ITLC/RTLC methods, for the purity. In the case of significant presence of impurities, the sample can be purified using gel filtration as described above. Control labeling experiments were also performed using $^{67}GaCl_3$, and DTPA with $^{67}GaCl_3$. Both reaction mixtures were passed through separate gel filtration columns and eluted with phosphate buffer solution (PBS). In case of gel filtration, the fractions which showed the presence of protein were used in other experiments ($n = 3$).

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

a. 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 1 mM DTPA in DDH_2O as the mobile phase.
b. High-performance liquid chromatography: HPLC was performed on the final preparation using acetate buffer solution (50 mM pH 5.5) as eluent (flow rate: 1 ml/min pressure: 130 KgF/cm^2) for 20 min in order to elute low molecular weight components. Radiolabeled peptide was eluted using a gradient of the latter solution (100 to 0%) and citrate buffer solution (50 mM, pH 4,0 to 100%) using reverse stationary phase. Any remaining free Ga^{3+} cation can be complexed with citrate anion, however precomplexed Ga-DTPA-insulin was not challenged with the addition of citrate chelate. At neutral pH, the binding constant for gallium citrate is approximately $\log K = 10.0$, and for gallium DTPA it is approximately $\log K = 25.7$ [9].

Stability testing of the radiolabeled compound

Stability of [^{67}Ga]-DTPA-insulin in PBS was determined by storing the final solution at 4°C for 14 days and performing ITLC analysis to determine radiochemical purity. ITLC analysis was performed frequently. Furthermore, the stability of the conjugated DTPA-insulin stored at –20°C for more than 1 month was investigated. ITLC analysis of the conjugated product was performed to monitor degradation products or other impurities. 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 human serum

Labeled compound stability in freshly prepared human 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 at room temperature; 0.5 mL fractions were collected.

Biodistribution of [^{67}Ga]-DTPA-insulin in normal rats

To determine its biodistribution, [^{67}Ga]-DTPA-insulin was administered to the normal rats (NMRI) purchased from Razi Institute, Karaj, Iran. A volume (50 µl)

of the final [^{67}Ga]-DTPA-insulin solution containing 40 ± 2 μCi radioactivity was injected intravenously to the rats through their tail vein. The animals were sacrificed at exact time intervals (3, 22, 44 and 110 h), and the specific activity of different organs was calculated as the percentage of urea under the curve of 184 keV peak per gram.

Results

Conjugation of insulin with DTPA cyclic dianhydride and radiolabeling of insulin with ^{67}Ga

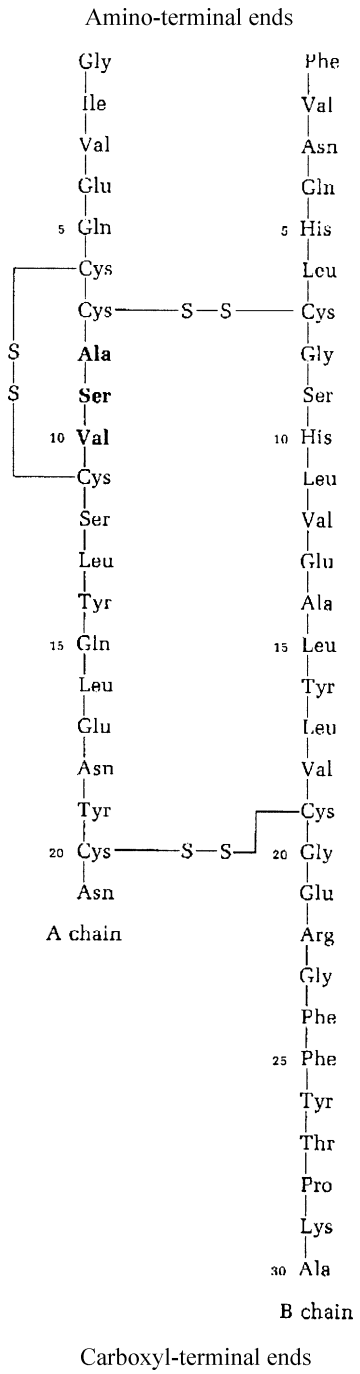

Fig. 1. Schematic diagram of insulin.

The labeling yield of ^{67}Ga-DTPA-insulin has been studied in the wide range of insulin/DTPA ratios in order to optimize the process and to improve the [^{67}Ga]-DTPA-insulin performance *in vitro*. The overall radiolabeling efficiency was over 77% and the specific activity was kept in the range of 300–500 MBq/mg. Because of its isoelectric point (IEP) of 5.3 to 5.4, insulin is soluble in weakly acid environments of pH 3 to pH 4, as well as in environments at pH 7 to pH 8 being adequately stable (Fig. 1). In the indicated pH ranges, the molecule may be positive (pH < IEP) or negatively charged (pH > IEP) [23].

The conjugated [^{67}Ga]-DTPA-insulin fractions containing the maximum protein content were mixed with ^{67}Ga-$GaCl_3$ solution, vortexed and kept at room temperature. Small fractions were taken from this mixture and tested by RTLC to find the best time scale for labeling. After an hour, the free ^{67}Ga/conjugated ^{67}Ga ratio in the labeled sample remained unchanged. The mixture was then passed through another Sephadex G-50 gel filtration column in order to remove trace amounts of unbound ^{67}Ga cation.

The eluted fractions were checked by the Folin-Colciteau® reagent and for the presence of radioactivity in order to determine the [^{67}Ga]-DTPA-insulin containing fractions. The fraction with a maximum absorbance using the folin method, which consisted of the maximum radioactivity as well, was chosen as a suitable final product with appropriate specific activity for animal tests. The radiolabeling reached to 90% after 60 min. Figure 2 demonstrates the RTLC scheme of free Ga^{3+}, Ga-DTPA and radiolabeled protein.

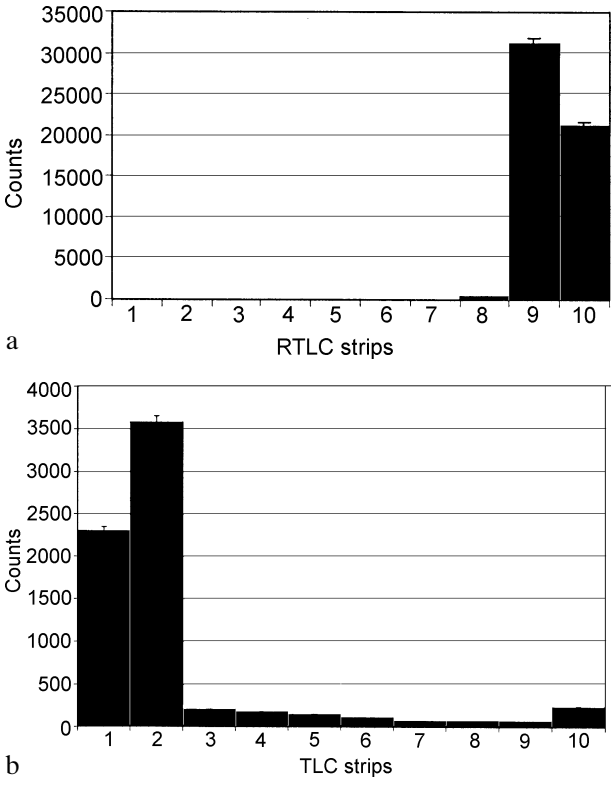

Fig. 2. RTLC chromatogram of $^{67}Ga^{3+}$, [^{67}Ga]-DTPA (a) and [^{67}Ga]-DTPA-insulin (b) on Whatman paper with 1 mM DTPA solution.

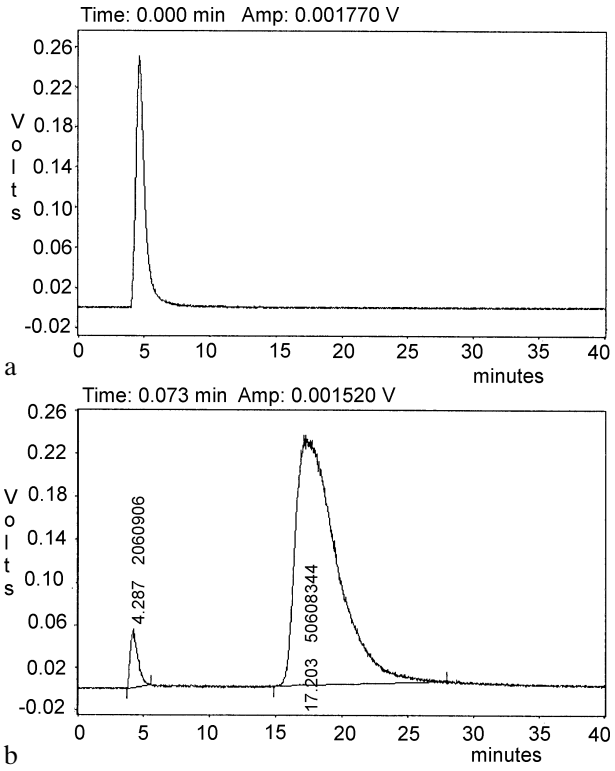

Fig. 3. HPLC chromatogram of control ^{67}GaDTPA solution (a) and final radiolabeled solution (b) on a reversed-phase column using a gradient of acetate/citrate buffer.

At this stage, the mixture was tested by HPLC in order to determine the radiochemical purity before administration to rodent models. Figure 3 shows the HPLC chromatogram of ^{67}GaDTPA and the final solution. The fast eluting component (4.28 min) was shown to be a mixture of free ^{67}Ga and ^{67}GaDTPA which were washed out on reversed-phase stationary phase. Both compounds are ionic, so they are eluted at the same retention time. The radiolabeled protein was finally washed out in 17.2 min.

Stability of radiolabeled protein *in vitro*

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 several hours, indicating that the Ga-protein chelate was of high stability.

These results were confirmed by gel filtration chromatography. After incubation of [^{67}Ga]-DTPA-insulin with PBS for 2 h, almost all of the radioactivity eluted in the same position as [^{67}Ga]-DTPA-insulin; there was no evidence for a large-scale release of free Ga. Similarly, gel filtration chromatography of [^{67}Ga]-DTPA-insulin 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 consistent with the normal blood clearance time of insulin.

Biodistribution studies

The distribution of [^{67}Ga]-DTPA-insulin among tissues were determined in the normal rats. A volume (0.1 ml) of [^{67}Ga]-DTPA-insulin solution containing 4.4–5.2 MBq radioactivity was injected into the dorsal tail vein. The total amount of radioactivity injected into each mouse was measured by counting a 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.

In 1–3 h a very low amount of activity is excreted via kidneys that could be due to the presence of small amounts of Ga^{3+} GaDTPA and/or minor dissociation of the protein conjugate. However, in the next few hours, the activity is rapidly removed form the blood and accumulated in the liver and slowly in the spleen as expected for a protein like insulin, after 44 h, the activity in the spleen reaches its maximum, while still the major organ of choice is the liver. After 110 h, the only major tissue of interest is the liver and the accumulated dose for other organs is negligible (Figs. 4–7).

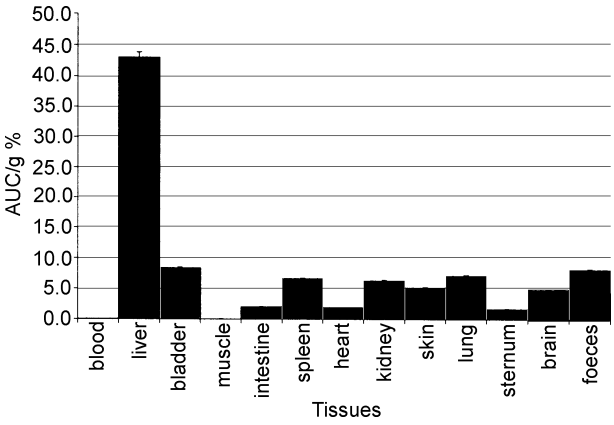

Fig. 4. Biodistribution of [^{67}Ga]-DTPA-insulin in normal rats 3 h postinjection. AUC: area under curve of the 184 keV peak in the gamma spectrum.

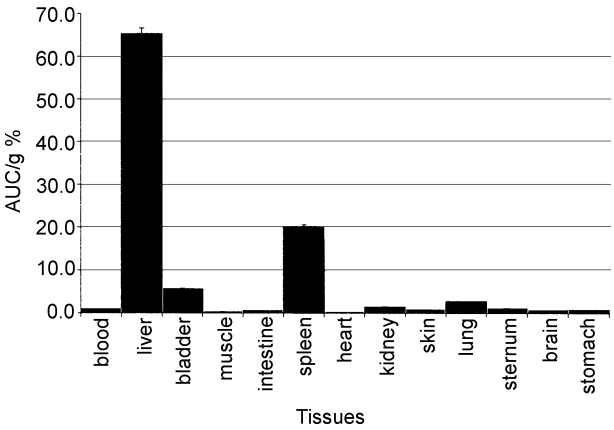

Fig. 5. Biodistribution of [^{67}Ga]-DTPA-insulin in normal rats 22 h postinjection. AUC: area under curve of the 184 keV peak in the gamma spectrum.

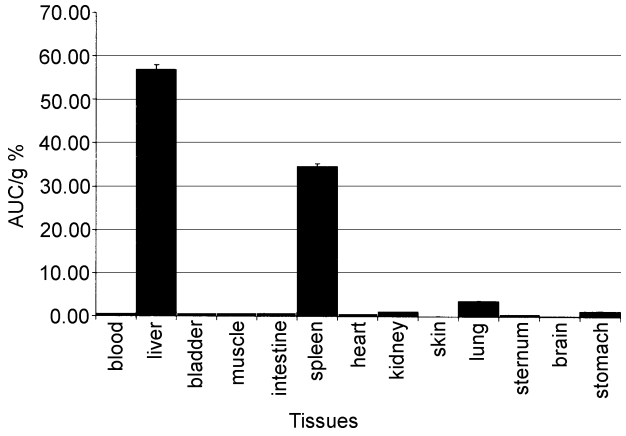

Fig. 6. Biodistribution of [^{67}Ga]-DTPA-insulin in normal rats 44 h postinjection. AUC: area under curve of the 184 keV peak in the gamma spectrum.

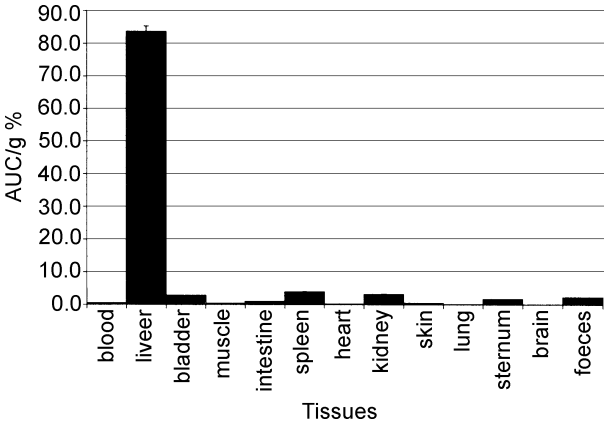

Fig. 7. Biodistribution of [^{67}Ga]-DTPA-insulin in normal rats 110 h postinjection. AUC: area under curve of the 184 keV peak in the gamma spectrum.

Discussion

The total labeling and formulation of [^{67}Ga]-DTPA-insulin took about 60 min. A suitable specific activity product was formed via insertion of the ^{67}Ga cation. No other labeled conjugates were observed upon RTLC and/or HPLC analysis of the final preparations. The radiolabeled complex was stable in the mice serum for at least 24 h and no significant amount of free ^{67}Ga as well as ^{67}Ga-DTPA was observed. A radiochemical purity higher than 96% was detected by HPLC. In 2–3 h the radiolabeled hormone is cleared from blood circulation and most of the tracer accumulates in the liver. This is in agreement with the reported ^{123}I-labeled insulin biodistribution results reported previously [2, 22]. Insulin:ccDTPA ratio of 1:1 was shown to be the best conjugation condition, suggesting that the only possible conjugation site could be the ^{29}Lys-NH_2 residue. [^{67}Ga]-DTPA-insulin is a good probe for biodistribution study of insulin receptors in diabetic conditions, hypoglycemic syndrome and possibly sugar consuming tumors.

Acknowledgment The authors wish to thank Ms Moradkhani and Mr S. Daneshvari for conducting animal studies.

References

1. Awasthi VS, Gambhir AB, Sewatkar B (1994) ^{99m}Tc-insulin: labeling, biodistribution and scintiimaging in animals. Nucl Med Biol 21:251–254
2. Dozio N, Sarugeri E, Scavini M *et al*. (2001) Insulin receptor antibodies inhibit insulin uptake by the liver: *in vivo* ^{123}I-insulin scintigraphic scanning and *in vitro* characterization in autoimmune hypoglycemia. J Investig Med 49:85–92
3. Dozio N, Scavini M, Beretta A *et al*. (1998) Imaging of the buffering effect of insulin antibodies in the autoimmune hypoglycemic syndrome. J Clin Endocrinol Metab 83:643–648
4. Eastman RC, Carson RE, Jacobson KE *et al*. (1992) *In vivo* imaging of insulin receptors in monkeys using ^{18}F-labeled insulin and positron emission tomography. Diabetes 41:855–860
5. Eckelman WC, Karesh SM, Reba RC (1975) New compounds: fatty acid and long chain hydrocarbon derivatives containing a strong chelating agent. J Pharm Sci 64:704–706
6. Firestone RB, Shirley VS (1996) Table of isotopes, 8th ed. John Wiley & Sons Inc., New York
7. Goethals P, Lemahieu I, Agon P (1991) ^{66}Ga – a promising isotope for positron emission tomography. Acta Radiol 37;Suppl:60–61
8. Graham MC, Pentlow KS, Mawlawi O, Finn RD, Daghighian F, Larson SM (1997) An investigation of the physical characteristics of ^{66}Ga as an isotope for PET imaging and quantification. Med Phys 24:317–326
9. Green MA, Welch MJ (1989) Gallium radiopharmaceutical chemistry. Nucl Med Biol 16:435–448
10. Guenther KJ, Yoganathan S, Garofalo R *et al*. (2006) Synthesis and *in vitro* evaluation of ^{18}F- and ^{19}F-labeled insulin: a new radiotracer for PET-based molecular imaging studies. J Med Chem 49:1466–1474
11. Hachiya HL, Treves ST, Kahn CR, Sodoyez JC, Sodoyez-Goffaux F (1987) Altered insulin distribution and metabolism in type I diabetics assessed by [^{123}I]insulin scanning. J Clin Endocrinol Metab 64:801–808
12. Hnatowich DJ, Layne WW, Child RL (1983) Radioactive labeling of antibody: a simple and efficient method. Science 220:613–619
13. Jalilian AR, Khorrami A, Tavakoli MB, Kamali-Dehghan M, Yari Kamrani Y (2007) Development of [^{201}Tl](III)-DTPA-human polyclonal insulin complex for inflammation detection. Radiochim Acta 95:669–675
14. Jalilian AR, Mirsadeghi L, Haji-Hosseini R (2007) Preparation and biodistribution of [^{67}Ga]-DTPA-rituximab in normal rats. J Radioanal Nucl Chem 274:175–179
15. Jalilian AR, Mirsadeghi L, Yari-Kamrani Y, Rowshanfarzad P, Kamali-Dehghan M, Sabet M (2007) Development of [^{64}Cu]-DOTA-anti-CD20 for targeted therapy. J Radioanal Nucl Chem 274:563–568
16. Jalilian AR, Rowshanfarzad P, Shafaii K *et al*. (2005) Development of ^{111}In-DTPA-human polyclonal antibody complex for long-term inflammation/infection detection. Nukleonika 50:91–96
17. Kasuga MY, Karlsson FA, Kahn CR (1982) Insulin stimulates the phosphorylation of the 95,000-dalton subunit of its own receptor. Science 215:185–187
18. Kasuga MY, Zick DL, Blithe FA, Karlsson HU, Kahn CR (1982) Insulin stimulation of phosphorylation of the beta subunit of the insulin receptor. Formation of both phosphoserine and phosphotyrosine. J Biol Chem 257:9891–9894

19. Lewis MR, Reichert DE, Laforest R *et al*. (2002) Production and purification of gallium-66 for preparation of tumor-targeting radiopharmaceuticals. Nucl Med Biol 29:701–706
20. Sabet M, Rowshanfarzad P, Jalilian AR, Ensaf MR, Rajamand AA (2006) Production and quality control of ^{66}Ga radionuclide. Nukleonika 51:147–154
21. Shai Y, Kirk KL, Channing MA *et al*. (1998) ^{18}F-labeled insulin: a prosthetic group methodology for incorporation of a positron emitter into peptides and proteins. Biochemistry 28:4801–4806
22. Sodoyez JC, Sodoyez-Goffaux F, von Frenckell R, De Vos CJ, Treves S, Kahn CR (1985) Differing effects of antiinsulin serum and antiinsulin receptor serum on ^{123}I-insulin metabolism in rats. J Clin Invest 75:1455–1462
23. Wunderlich JC, Schick U, Freidenreich J, Werry J (1994) Peroral dosage form for peptide containing medicaments, in particular insulin. United States Patent: 6,468,959 Issued: October 22, 2002 Appl. No 222714
24. Zweit J, Sharma H, Downey S (1987) Production of gallium-66, a short-lived, positron emitting radionuclide. Int J Radiat Appl Instrum A 38:499–501