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Original research paper

Development of a radiolabeled β-human chorionic gonadotropin

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β-Human chorionic gonadotropin (β-hCG) was successively 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 showed radiochemical purity higher than 95 % under optimized conditions (specific activity = 22–23 TBq mM^{-1}, labeling efficiency 80 %). Preliminary *in vivo* studies (ID g^{-1}, %) in male wild-type rats showed marked gonadal uptake of the tracer after 240 minutes in agreement with the biodistribution studies and reported β-hCG receptors. Target to blood ratios were 5.1 and 15.2 after 3 and 24 hours, respectively, while target to muscle ratios were 35 and 40 after 3 and 24 hours, respectively.

Keywords: β-hCG, radiolabeling, biodistribution, radiopharmaceutical

Human chorionic gonadotropin (hCG) is a glycoprotein hormone produced in pregnancy by the developing embryo after conception and later by the syncytiotrophoblast to prevent disintegration of the corpus luteum of the ovary. hCG also plays a role in cellular differentiation/proliferation and may activate apoptosis (1). hCG is extensively used as a parenteral fertility medication in lieu of luteinizing hormone.

hCG is composed of 244 amino acids with a molecular mass of 36.7 kDa. It is heterodimeric, with α (alpha) and β (beta) subunits creating a small hydrophobic core. The vast majority of the outer amino acids are hydrophilic.

hCG has been radiolabeled mostly with ^{125}I for various applications, such as kinetic studies of hCG conformations (2), thermodynamic analysis of hCG monoclonal antibodies (3), structure-activity studies (4) and placental protein synthesis studies (5).

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hCG receptors (hCGRs) are mainly located on the gonads, and also on many other tissues including female and male breasts (6). hCGR is also widely overexpressed in various neoplasmic cells (7), human colon malignancies (8), ovarian, endometrial, and uterine cervix cancers (9), and 20–40 % of all common epithelial carcinomas (10).

It is used in the development of targeting agents for cancer therapy (11) and has been shown to be highly effective in targeting and destroying prostate and breast cancer cell metastases *in vitro* (12). hCGR has been also used in targeted delivery of superparamagnetic iron oxide nanoparticles for MRI studies of metastatic breast cancer cells in peripheral tissues (13) and as a conjugated form with doxorubicin (14).

According to our knowledge, there are no reports of radiolabeled hCG hormone and its truncates for imaging studies in the literature. In this work, following the preparation of an hCG conjugate for diagnostic hCGR studies using metallic positron emission tomography (PET) or single photon computed tomography (SPECT) radioisotopes, ^{67}Ga-labeled hCG was prepared for preliminary biodistribution studies, based on our recent experience of the preparation of radiometal-labeled proteins (15).

A precise labeling strategy was employed using freshly-prepared diethylenetriamine--pentaacetic dianhydride (ccDTPA), at various hCG concentrations and gallium-67 for optimization of radiolabeling conditions due to its longer half-life compared to PET gallium radionuclides.

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 with > 95 % enrichment was obtained from the Ion Beam Separation Department at AMIRS. All chemicals were purchased from commercial sources. Pregnyl™, (β-hCG), is a pharmaceutical sample purchased form Daro-Pakhsh Co., Iran (1500 IU) and was used without further purification. Cyclic DTPA dianhydride was freshly prepared and kept under a blanket of N_2. Paper chromatography (PC) was performed by counting Whatman No. 2 paper (Whatman, UK) using a thin layer chromatography scanner, Bioscan AR2000, Bioscan Europe Ltd. (France). To determine the specific activity, analytical HPLC was performed using a Shimadzu LC-10AT (Japan) instrument, armed with two detector systems, flow scintillation analyzer (Packard-150 TR) and UV-visible detector (Shimadzu), and 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. Approval of the AMIRS Ethical Committee was obtained for this research.

The wild-type rats (NMRI) were purchased from the Pasteur Institute of Iran, Karaj, all weighing 180–200 g and were acclimatized at proper rodent diet and 12h/12h day/night cycle.

Percentage of the injected dose in tissue (ID g^{-1}, %) was determined using a high purity germanium (HPGe) detector coupled with a Canberra^{TM} (model GC1020-7500SL, USA) multichannel analyzer based on the area under the curve for 184 keV photopeak and the calculated counting system efficiency.

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 the radiochemical separation process. After the target bombardment process, chemical separation was carried out in the 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 37–74 μm), which had been pre-conditioned by passing 9 mol L^{-1} HCl. The column was then washed with 25 mL of 9 mol L^{-1} HCl at a rate of 1 mL min^{-1} to remove copper and zinc ions. To the eluent, 30 mL water plus about 100 mL of 6 mol L^{-1} HCl solution was added. The latter solution was loaded on another exchange resin (AG1X8 Cl^- form, 74–149 μm) pretreated with 6 mol L^{-1} HCl (100 mL). Finally, 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 in a high purity germanium detector coupled to a Canberra^{TM} multi--channel analyzer for 1000 seconds.

Chemical purity control. – The presence of zinc and copper cations was checked by polarography. The area under the curve of the polarogram of test samples was lower than that of the standards even at 1 μg mL^{-1} of standard zinc and copper.

Conjugation of ccDTPA with human recombinant hCG. – The chelator ccDTPA was conjugated to the hCG using slightly modified cyclic anhydride method (16). Conjugation was performed at a 1:1 molar ratio. In brief, 20 μL of a 1 mg mL^{-1} suspension of ccDTPA 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 hCG (1500 IU, 1 mL, pH 8, ≈ 3 nmol) (17) was subsequently added and gently mixed at room temperature for 60 min.

Radiolabeling of hCG conjugate with ^{67}Ga. – The hCG conjugate was labeled using an optimized protocol according to the literature (18). Typically, 74 MBq of ^{67}Ga-chloride (in 0.2 mol L^{-1} HCl) was added to a conical vial and dried under a nitrogen flow. To the ^{67}Ga containing vial, the conjugated fraction was added in 1 mL of phosphate buffer (0.1 mol L^{-1}, pH 8) and mixed gently for 30 seconds. The resulting solution was incubated at room temperature for 30 minutes. Following incubation, the radiolabeled hCG conjugate was checked using PC methods for purity. In case of the presence of unreacted amounts of impurities, the sample can be purified by solid phase extraction using C_{18} Sep-Pak. Briefly, the column was pretreated with absolute ethanol and water, respectively, followed by injection of the radiolabeling mixture. The column was left at room tempera-

ture for 5 min and was then washed with water fractions until 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 ccDTPA with $^{67}GaCl_3$.

Quality control of [^{67}Ga]-hCG. – A 5-μL sample of the final fraction was spotted on a chromatography paper (Whatman No. 1, Whatman), and developed in a mixture of 1 mol L^{-1} ccDTPA in H_2O as the mobile phase.

High performance liquid chromatography. – HPLC was performed on the final preparation using reverse stationary phase. Acetate buffer solution (50 mmol L^{-1} pH 5.5) was eluent A and citrate buffer solution was B (50 mmol L^{-1}, pH 4) (flow rate 1 mL min^{-1}) for 20 min in order to elute low molecular mass components. Radiolabeled peptide was eluted using the following gradients: 100 % A, 0 % B 5 min, 70 % A, 30 % B 5 min, 50 % A, 50 % B 5 min, 0 % A, 100 % B 5 min.

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

Stability testing of the radiolabeled compound in the presence of serum. – Labeled compound stability in serum was assessed by gel filtration on a Sepharose column. The column was equilibrated with PBS and eluted with PBS at a flow rate of 0.5 mL min^{-1} at room temperature; 0.5 mL fractions were collected.

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

RESULTS AND DISCUSSION

The labeling yield of ^{67}Ga-DTPA-hCG has been studied in a wide range of hCG/DTPA ratios in order to optimize the process and to improve ^{67}Ga-DTPA-hCG performance *in vitro*. The overall radiolabeling efficiency was over 80 %. Because of its isoelectric point (IEP) of around 4, hCG is soluble in physiological serum pH (5.5–7) being adequately stable (19). However, its structural integrity is strongly dependent on the disulphide bonds, and its reduction completely destroys the structure; thus, the radiolabeling strategies based on redox reactions cannot be employed.

Regarding the peptide sequence for hCG for alpha and beta subunits, the existence of 6 lysine moieties in the alpha subunit and 4 in the beta subunit, can load to NH_2-mediated conjugation through ccDTPA acylation.

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

For better radiochemical purity, solid-phase extraction using a C_{18} column was used. The radiolabeled mixture was loaded on the pre-conditioned Sep-Pak Columm. Eluting the loaded column with water removed free $^{67}Ga^{3+}$ as well as ^{67}Ga-DTPA due to their ionic properties. After purging the column with nitrogen for 5 minutes, the radiolabeled protein was eluted using citrate buffer in the first 3 elutions. At this stage, the buffer eluted fraction with the highest activity was tested by HPLC in order to determine its radiochemical purity before administration to wild-type rats for biodistribution studies. Fig. 1a shows the HPLC chromatogram of $^{67}Ga^{3+}$. The fast eluting component (2.79 min) was shown to be a mixture of free ^{67}Ga and ^{67}Ga-DTPA which was washed out on the reverse phase stationary phase. The radiolabeled protein was washed out in 14.78 minutes (Fig. 1b).

It has been already shown that the median *in vivo* and *in vitro* biopotency for hCG is approximately 13000 IU mg^{-1}, with a range from 8360 to 16800 IU mg^{-1} (22). Thus, considering the used (74 MBq) and the radiochemical purity of the final purified sample (95 %), a specific activity of 22–23 TBq mmol^{-1} was obtained after Sep-Pak purification.

Stability of the radiolabeled protein *in vitro* was determined after challenge with phosphate-buffered saline and serum. PC 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-hCG with PBS for 2 h there was no change in the R_f for [^{67}Ga]-DTPA-hCG

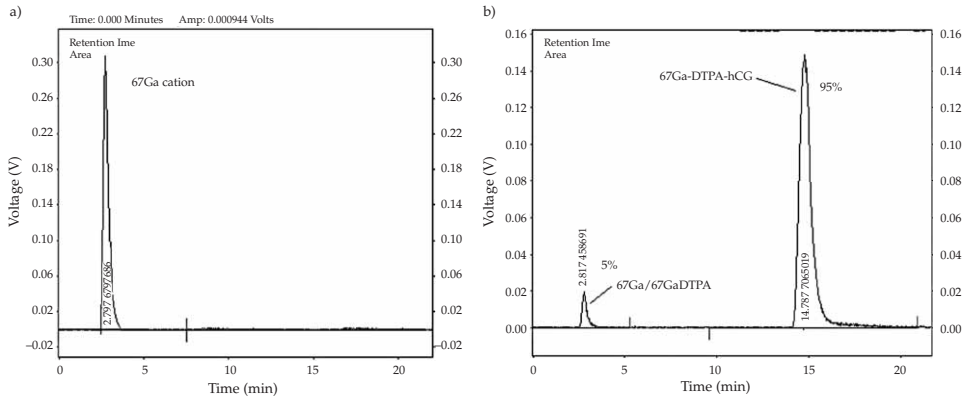

Fig. 1. HPLC chromatogram of: a) free $^{67}GaCl_3$ solution, b) of solid phase purified final radiolabeled solution, on a reversed phase column using a gradient of acetate/citrate buffer.

and there was no evidence of any large-scale release of free Ga resulting in the appearance of a new radiopeak. Similarly, gel filtration chromatography of [^{67}Ga]-DTPA-hCG after a 2-h incubation with human serum showed that the radioactivity still eluted in the same fraction. Thus, there was no evidence of either degradation or transchelation of ^{67}Ga to other serum proteins over a time period.

The distribution of free $^{67}GaCl_3$ in appropriate buffer has been already reported elsewhere (21) Fig. 2 demonstrates the biodistribution of [^{67}Ga]-DTPA-hCG among tissues in male wild-type rats. A volume (0.1 mL) of final [^{67}Ga]-DTPA-hCG solution containing 40 μCi of radioactivity was injected into the dorsal tail vein. The total amount of radioactivity injected into each rat was determined by measuring the 1-mL syringe before and after injection in a dose calibrator with fixed geometry.

The animals were sacrificed by ether asyxphycation at selected times after injection (0.25 h to 2 h), the tissues (blood, heart, spleen, kidneys, liver, intestine, muscle, bone, brain, stomach, lung, skin, pancreas, bladder and testes) were weighed and their specific activities were determined with a γ-ray scintillation detector as percent of the area under the curve of 184 keV per gram of tissue.

The radiolabeled hormone is gradually removed from the circulation and possible serum protein binding exists for this hormone. Most non-glycosylated proteins mainly possess hepatic accumulation, while due to the existence of residual sugar moieties on the hCG structure (leading to more solubility) liver uptake is not significant in this case.

Skin, muscle, brain pancreas and also kidney demonstrated low uptake; however, spleen demonstrated high uptake. Up to 120 minutes the testes showed low uptake while after 240 minutes 16 % of the tracer accumulated in the testes.

hCG transport in rat testicular microvasculature has been already studied by electron microscopy and by analyzing the transfer of the radiolabeled hormone. It has been observed that the same receptor molecule which is present in target Leydig cells is also

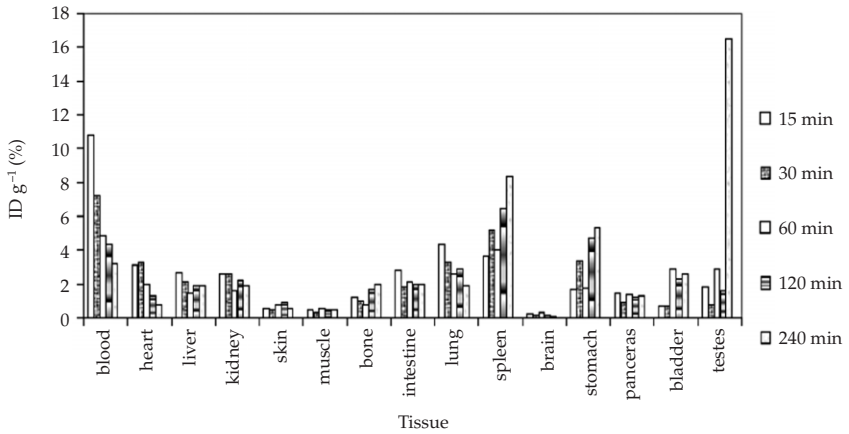

Fig. 2. Biodistribution of [^{67}Ga]-DTPA-hCG (1.85 MBq, 40 μCi) in wild-type rats 0.25–24 h after *i.v.* injection *via* tail vein. (ID g^{-1} (%) – percentage of injected dose per gram of tissue based on the area under the curve of 184 keV peak in gamma spectrum.

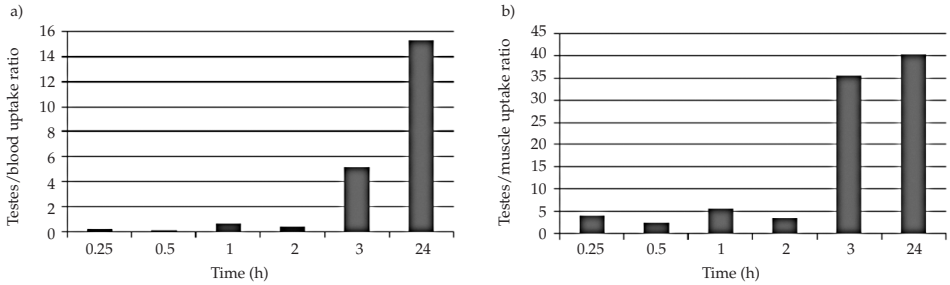

Fig. 3. a) Target/blood and b) target/muscle ratios for [^{67}Ga]-DTPA-hCG 0.25–24 h after administration.

involved in transcytosis through the endothelial cells (22). The hormone can be internalized by coated pits and vesicles on the luminal side of the endothelium. It was then localized in the endosomal compartment and subsequently appeared to be delivered by smooth vesicles into the subendothelial space.

hCG receptors are widely expressed in gonadal cells; however, the presence of these receptors was also detected in several other non-gonadal female and male tissues. The expression level of non-gonadal hCG receptors is much lower than in the gonads, although their expression is regulated by similar mechanisms and observed in malignancies (20).

For better comparison, the target/blood and target/muscle ratios were determined from ID g^{-1} (%) curves to demonstrate the time dependence of specific uptake in the target organ. The significance of uptake in gonadal tissue was observed at least 3 hours after administration of the tracer in both cases. However, the hCG hormone partly remained in serum binding species while after 24 hours major accumulation with the lowest background was in the testes (Fig. 3).

CONCLUSIONS

Total labeling and formulation of [^{67}Ga]-DTPA-HCG took about 60 minutes. A suitable specific activity product was formed *via* insertion of ^{67}Ga cation. No other labeled conjugates were observed upon PC 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 or ^{67}Ga-DTPA was observed. A 95 % radiochemical purity was detected by HPLC. The final preparation was administered to wild-type rats and biodistribution of the radiopharmaceutical was checked 0.25 to 24 hours later. In 2–3 hours, the radiolabeled hormone was cleared from the blood circle and most of the tracer accumulated in spleen. After 3–24 h, major tracer uptake was observed in the testes. Target/muscle ratio after 24 h reached 40, demonstrating specific accumulation on the hCGR positive tissues. [^{67}Ga]-DTPA-hCG can be a suitable probe for biodistribution of hCGR in various physiological and malignant diseases with over-expressed hCGRs.

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SAŽETAK

Razvoj radiomarkiranog β-humanog koriogonadotropina

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β-humani korionski gonadotropin (β-hCG) uspješno je markiran s [^{67}Ga] galijevim kloridom nakon konjugacije sa svježe priređenim dianhidridom dietilentriaminpentaoctene kiseline (ccDTPA). Nakon čišćenja radiomarkiranog hormona na čvrstoj fazi, radiokemijska čistoća bila je prema HPLC veća od 95 % (specifična aktivnost = 22–23 TBq mM^{-1}, učinkovitost markiranja 80 %). Preliminarni *in vivo* pokusi (ID g^{-1}, %) na mužjacima divljeg tipa štakora pokazali su da obilježeni hormon značajno ulazi u gonade nakon 240 minuta, što je u suglasnosti s ispitivanjima biodistribucije i podacima o receptorima za β-hCG. Omjer koncentracija u gonadama i krvi bio je 5,1, odnosno 15,2 nakon 3, odnosno 24 sata, dok je omjer koncentracija u gonadama i mišićima bio 35, odnosno 40 nakon 3, odnosno 24 sata.

Keywords: β-hCG, radiomarkiranje, biodistribucija, radiofarmaka

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