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Production and evaluation of [^{67}Ga]-DTPA-Rituximab

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Background: In order to obtain an anti-CD20 conjugate to be used in future therapeutic studies with therapeutic radioisotopes, ^{67}Ga-labeled antibody was prepared as a model of metal chelated immunoconjugate for preliminary dosimetric and biodistribution studies. ***Materials and Methods:*** Rituximab was labeled with [^{67}Ga]-gallium chloride after residulation with freshly prepared cyclic DTPA-dianhydride. The best results of the conjugation were obtained by the addition of 1 ml of a rituximab pharmaceutical solution (5 mg/ml, in phosphate buffer, pH=8) to a glass tube pre-coated with DTPA-dianhydride (0.01 mg) at 25°C with continuous mild stirring for 30 min. The final isotonic ^{67}Ga-DTPA-rituximab complex was checked by gel electrophoresis for radiolysis/chemolysis control. Radio-TLC was performed to ensure the formation of only one species. Preliminary *in vivo* studies in normal rat model were performed to determine the biodistribution of the radioimmunoconjugate up to 6 hours. ***Results:*** Radio-thin layer chromatography showed an overall radiochemical purity of 96-99% at optimized conditions (specific activity =300-500 MBq/mg, labeling efficiency 77%). Gel electrophoresis showed no protein cleavage after radiolabeling. ***Conclusion:*** Preliminary *in vivo* studies in normal rat model showed [^{67}Ga]-DTPA-rituximab is a good probe for bio-dosimetry of therapeutic rituximab conjugates.
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Keywords: *Gallium-67, Rituximab, radiolabeling, biodistribution, cyclotron.*

INTRODUCTION

Rituximab is a chimeric mouse-human monoclonal antibody. Rituximab, selectively, binds with high affinity to the CD20 antigen (human B-lymphocyte-restricted differentiation antigen, Bp35), a hydrophobic transmembrane protein, which is expressed on B-lymphocytes and on more than 90% of B cell non-Hodgkin's lymphomas. This antigen regulates the early step(s) in the activation process for cell cycle initiation and differentiation [1]. CD20 is not shed from the cell surface and does not internalize upon antibody binding. Free CD20 antigen is not found in the circulation. Rituximab is thought to deplete CD20-positive cells via antibody-dependent cell-cytotoxicity and complement mediated cell lysis. Several studies of radiolabeled anti-CD20 monoclonal antibodies at nonmyeloablative doses in treating B-cell NHL have been reported, and several others are in progress. The agents for which most data are available are ^{131}I-tositumomab (Bexxar) [2] and yttrium ^{90}Y-ibritumomab tiuxetan (Zevalin) [3]. These studies have reported response rates of 25% to 40% with median response duration of 6 to 18 months in most studies and some very durable responses of more than 5 years [4-7]. In some studies rituximab has been labeled for metabolism and localization of CD20 antigens throughout body and/or penetration of the antibody to specific organs [8].

In order to obtain an anti-CD20 conjugate to be used in future therapeutic studies using therapeutic radioisotopes, ^{67}Ga-labeled antibody was prepared as a model of metal chelated immunoconjugate for preliminary dosimetric and biodistribution studies. Based on our recent experiences on the preparation of radiometal-labeled antibodies in our group [9], we were interested in the preparation of cyclotron-derived antiCD-20 immunoconjugate. A precise labeling strategy was employed

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using freshly-prepared DTPA cyclic dianhydride, with various rituximab concentrations. Finally, an optimized radiolabeling method for developing a highly reactive DTPA-conjugated anti-CD20 for possible radiometal studies has been has been introduced.

MATERIALS AND METHODS

Production of ^{67}Ga was performed at the Nuclear Research Center for Agriculture and Medicine (NRCAM) 30 MeV cyclotron (Cyclone-30, IBA). Enriched zinc-68 chloride with enrichment of >95% was obtained from 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. (U.K.).

Cyclic DTPA dianhydride was freshly prepared and kept under a blanket of N_2. Rituximab was a pharmaceutical sample purchased from Roche Co., and was used without further purification. ^{1}H-NMR spectra were obtained on a FT-80 (80MHz) Varian instrument with tetramethylsilane as the internal standard. Infrared spectra were taken on a Perkin-Elmer 781 instrument (KBr disc). Thin layer chromatography (TLC) of non-radioactive products was performed on polymer-backed silica gel (F 1500/LS 254, 20×20 cm, TLC Ready Foil, Schleicher & Schuell®).

Mixtures of ammonium acetate/10%-methanol (50:50 or 90:10) were used as eluent. 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) multi-channel analyzer. 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 student *t-test*. Statistical significance was defined as $P<0.05$.

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 have been 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, Φ:1.3 cm) which had been preconditioned by passing 25 ml of 9 M HCl. The column was then washed by 25 ml of 9M HCl at a rate of 1 ml/min to remove copper and zinc ions. 30 ml water plus about 100 ml of a 6 M HCl solution was added to the eluent. The latter solution was loaded on another exchange resin (AG1X8 Cl^- form, 100-200 mesh, h: 25 cm, Φ: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.

Preparation of fresh cyclic DTPA dianhydride for optimal protein residulation

The compound was prepared according to the standard methods with slight modifications [10]. Briefly, DTPA in acidic form (0.1 mole) was heated with a 4-fold molar excess of acetic anhydride (0.4 mole), dissolved in 50 ml of pyridine and heated at 65°C for 24 h. The resulting anhydride was insoluble in pyridine and was collected by filtration, purified by repeated washing with acetic anhydride, and with anhydrous ether at the end. Drying in an oven at 50-60°C removed the last traces of solvent. The melting point was 178-180°C. ^{1}H NMR and IR spectra were in accordance with the literature.

Conjugation of cyclic DTPA di-anhydride with the Rituximab

The chelator diethylenetriamine penta-acetic acid dianhydride, prepared above, was conjugated to the antibody using a small modification of the well-known cyclic anhydride method [10]. 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 pipette under ultrasonication and transferred to a glass tube. The chloroform was evaporated under a gentle stream of nitrogen. Commercially available rituximab (5 mg, 0.5 ml, pH 8) was subsequently added and gently mixed at room temperature for 60 min. 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 immunoconjugate were chosen and kept at 4°C and for radiolabeling.

Radiolabeling of the antibody conjugate with ^{67}Ga

The antibody conjugate was labeled using an optimization protocol according to literature [12]. Typically, 37-40 MBq of ^{67}Ga-chloride (in 0.2M HCl) was added to a conical vial and dried under a flow of nitrogen. Conjugated fraction was added in to the Ga containing vial 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 the incubation, the radiolabeled antibody conjugate was purified from free ^{67}Ga by gel filtration on a Sephadex G-50 column (15-20 ml bed volume), and eluted with PBS. Fractions (1 ml) were collected and the radioactivity of each fraction was measured by a recently calibrated radioisotope dose calibrator (CRC-7, Capintec Instruments, Ramsey, NJ). The protein presence in each fraction was determined using a fast protein assay method by mixing freshly prepared Folin-Colciteau® reagent, and 10 µl of the eluted fractions. The fractions containing the proteins with the maximum radioactivity were combined and tested for purity by ITLC, using a radio TLC scanner. 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 PBS. Fraction numbers 5-7 showed the presence of protein, and fraction 6 was used in the other experiments (n=3).

Quality control of ^{67}Ga-DTPA-rituximab

a. Thin layer chromatography

System I: A 5 µl sample of the final fraction was spotted on a silica gel paper and developed in a mixture of ammonium acetate (10%):methanol (9:1) as the mobile phase, in order to observe the R_f values of free $^{67}Ga^{3+}$ and ^{67}Ga-DTPA (0.5 and 0.9), while radiolabeled protein stays at the bottom (R_f= 0.0).

System II: Another system was used on silica-impregnated glass fiber sheets. From the final product, 5 µl was applied to the ITLC strip that was developed with 0.9% NaCl for 5 min. Radioactivity was determined by a chromatography scanner equipped with an HPGe crystal. ^{67}Ga-DTPA moved to the front, the ^{67}Ga-labelled monoclonal antibody remained at the starting position.

b. Paper chromatography

Paper chromatography [Whatman No. 1 (Whatman, Maidstone, UK), methanol/water (55:45)] of the elute showed that >94% of the activity remained at the origin, corresponding to the ^{67}Ga-DTPA-conjugate. The labeling yield was 45±5% (n=3), and a specific activity of 300-500 MBq per 1 mg DOTA-conjugate was obtained.

c. Radiolabeled Antibody integration

Gel electrophoresis was performed in order to evaluate possible radiolysis/chemolysis of rituximab in the course of reaction and purification. The samples of rainbow protein ladder standard, as well as pure unlabeled rituximab and ^{67}Ga-DTPA-conjugate were loaded over a 16% bis-acrylamide /acrylamide gel, followed by running at the constant voltage of 285V for 40 min. The current was then disconnected and the gels were stained in bromphenol blue for 3 hours followed by de-staining with MeOH acetic acid mixture. In the case of diluted protein samples, silver

staining of the gel was employed.

Stability testing of the radiolabeled compound

Stability of ^{67}Ga-DTPA-rituximab in PBS was determined by storing the final solution at 4°C for 14 days and performing frequent ITLC analysis to determine radiochemical purity. Frequent ITLC analysis was performed. Furthermore, the stability of the conjugated DTPA-rituximab stored at -20°C for more than 1 year was investigated. ITLC analysis of the conjugated product was performed to monitor degradation products or other impurities. After subsequent ^{67}Ga-labelling 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 at room temperature: 0.5 mL fractions were collected.

Biodistribution of ^{67}Ga-DTPA-rituximab in normal rats

To determine its biodistribution, ^{67}Ga-DTPA-rituximab was administered to normal rats. A volume (50-100 µl) of final ^{67}Ga-DTPA-rituximab solution containing 20±5 µCi radioactivity was injected intravenously to rats through their tail vein. The animals were sacrificed at exact time intervals (2, 6 and 24h), and the specific activity of different organs was calculated as percentage of injected dose per gram using a radiometer.

RESULTS

Preparation and structure confirmation of DTPA cyclic di-anhydride

In order to prepare the bi-functional ligand, DTPA cyclic *di*-anhydride (which was not cost effective) we tried the general procedure for its preparation [10]. The reaction was performed in pyridine containing DTPA acid form and acetic anhydride. The filtered mass was washed with cold acetic anhydride to remove residues of the reactant. The solid was dried in an oven for a couple of hours, and finally re-crystallized to get a high purity product, suitable for spectroscopic and radiolabeling steps (figure 1). Washing/drying steps were very important in that more repetition of these steps afforded high-purity product with rather long shelf-life. Such samples can be stored at room temperature under a blanket of N_2 for up to one year.

Conjugation of rituximab with DTPA cyclic di-anhydride and radiolabeling of rituximab with ^{67}Ga

The eluted fractions were checked by Folin-Colciteau® reagent, and for presence of radioactivity in order to determine the ^{67}Ga-DTPA-rituximab containing fractions.

Figure 2 shows the radioactivity content of the eluted fractions, as well as the protein presence. Fraction number 6 was chosen as the suitable final product with appropriate specific activity for animal tests.

Stability of radiolabeled protein in vitro

These results were confirmed by gel filtration chromatography. After incubation of [^{67}Ga]-DTPA-rituximab with PBS for 2 hours,

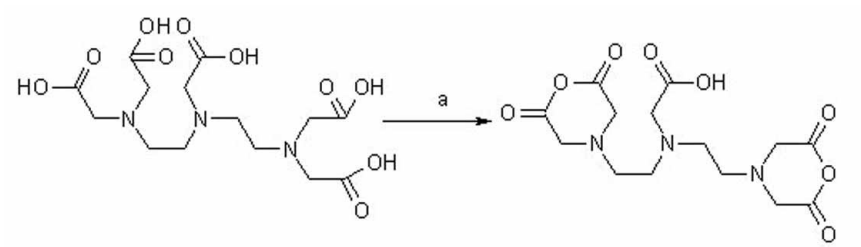

Figure 1. Schematic diagram of the synthesis of DTPA cyclic di-anhydride.

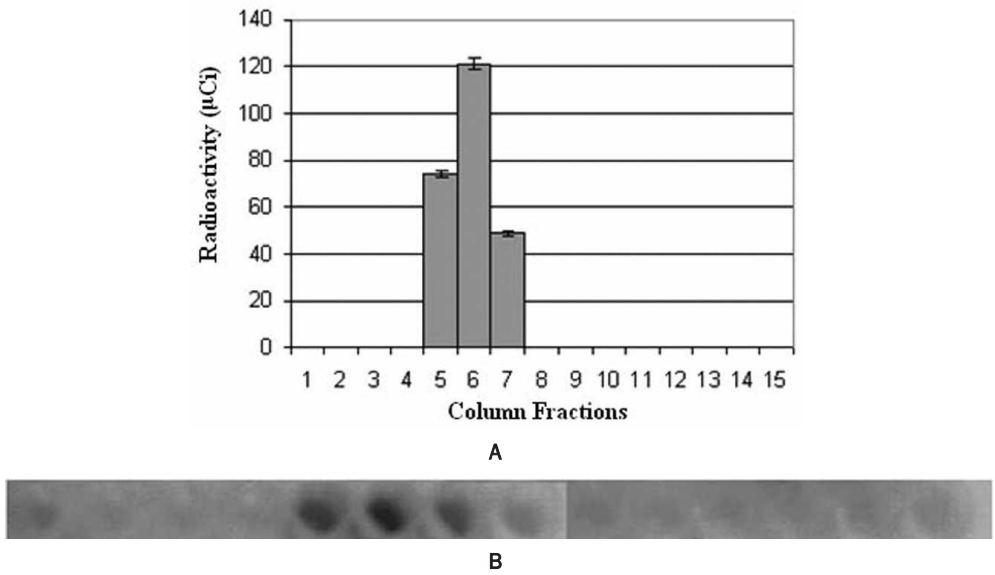

Figure 2. Radioactivity (A) (measured by dose calibrator) & colorimetric (B) assay (folin colciteau method) of ^{67}Ga-DTPA-rituximab fractions eluted from gel filtration column.

almost all of the radioactivity eluted in the same position as [^{67}Ga]-DTPA-rituximab: there was no evidence for large-scale release of free Ga. Similarly, gel filtration chromatography of ^{67}Ga-DTPA-rituximab 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 rituximab.

Biodistribution studies

The distribution of [^{67}Ga]-DTPA-rituximab among tissues were determined for untreated rats, and for rats with inflammatory lesions. A volume (0.1 ml) of final [^{67}Ga]-DTPA-rituximab solution containing 4.4-5.2 MBq radioactivities (≤6 µg IgG in 100 µL) was injected into the dorsal tail vein. The total amount of radioactivity injected into each mouse was measured by counting the 1-ml syringe before and after injection, in a curiemeter with a fixed geometry. The animals were sacrificed by ether asyxphycation at selected times after injection (2, 6 h), the tissues (blood, heart, spleen, kidneys, liver, intestine, stomach, lung, skin) and feces were weighed and their specific activities were determined with a γ-ray scintillation as a percent of the injected dose per gram of tissue (figures 3 and 4).

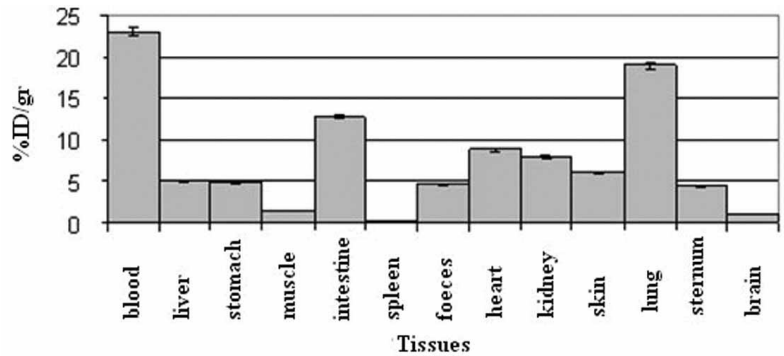

Figure 3. Bio-distribution of ^{67}Ga-DTPA-rituximab in normal rats 3 h post-injection (ID/g%: area under curve of 184 keV peak in gamma spectrum/gram of organ %).

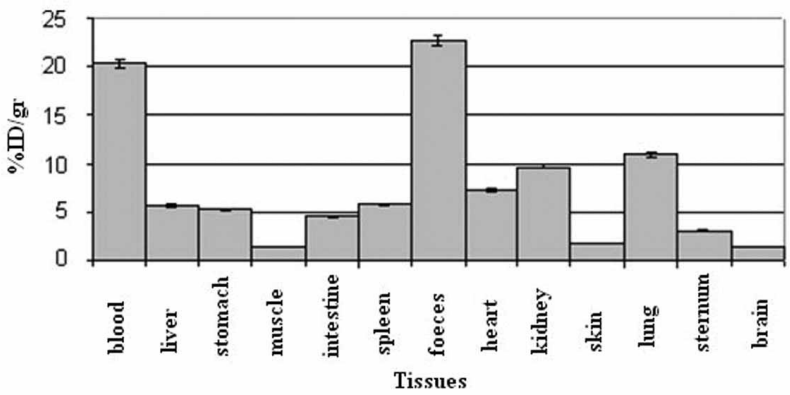

Figure 4. Bio-distribution of ^{67}Ga-DTPA-rituximab in normal rats 6 h post-injection (ID/g%: area under curve of 184 keV peak in gamma spectrum/gram of organ %).

DISCUSSION

The aim of this study was to explore the possibility of radiolabelling rituximab with radiogallium for use as an imaging agent in NHL for early detection, staging, remission assessment, monitoring for metastatic spread and tumor recurrence, and assessment of CD20 expression prior to (radio) immunotherapy.

The labeling yield of ^{67}Ga-DTPA-rituximab has been studied in the wide range of antibody/DTPA ratios in order to optimize the process and to improve ^{67}Ga-DTPA-rituximab performance *in vitro*. The overall radiolabeling efficiency was over 77%, and the specific activity was kept in the range of 300-500 MBq/mg.

The conjugated ^{67}Ga-DTPA-rituximab 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, 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 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 affinity. The biodistribution of the tracer in the animals were shown upto 6 hours post injection.

Total labeling and formulation of [^{67}Ga]-DTPA-rituximab took about 60 minutes, with a yield of 99%. A suitable specific activity product was formed *via* insertion of ^{67}Ga cation. No unlabelled and/or labeled conjugates were observed upon RTLC analysis of the final preparations. The radio-labeled complex was stable in mice serum for at least 24 hours, and no significant amount of free ^{67}Ga, as well as ^{67}Ga-DTPA was observed.

Trace amounts of ^{67}Ga-gallium chloride ($\backsimeq$1%) were detected by TLC. The final preparation was administered to normal rats, and biodistribution of the radiopharmaceutical was checked 1 and 6 hours later. In most of 40 rats tested, accumulation in the lungs was observed. At the beginning it was concluded that this accumulation is caused by the non-specific immigration of the lymphocytes to the possibly infected bronchi of the objects which the infection. But the infection was not later confirmed by the lab tests post-mortem. Interestingly, we found reports of severe pulmonary reactions with pulmonary infiltrates or edema in human. Acute symptoms appear within 1-2 hours of the initiation of the 1st infusion [11].

Since there has been no report on the production of gallium-DTPA-rituximab by our knowledge, this data can not be directly compared with the reported data. However, high GI and blood activity is not consistent with other radiolabeled antibodies already reported. Most of radiolabeled antibodies show high retention in liver instead of blood and GI in the first few hours.

Recent reports on SPECT radiolabeled rituximabs [12] have shown satisfactory

results, however, no biodistribution study was reported on trivalent Rituximab bioconjugates.

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