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ORIGINAL PAPER

Development of ^{153}Sm-DTPA-rituximab for radioimmunotherapy

Ali Bahrami-Samani,
Mohammad Ghannadi-Maragheh,
Amir R. Jalilian,
Hassan Yousefnia,
Javad Garousi,
Sedigheh Moradkhani

Abstract. Combining beta-particle effect with therapeutic properties of anti-CD20 monoclonal antibody in lymphomas, Mabthera™ (rituximab) was targeted in this study. The antibody was labeled with ^{153}Sm-samarium chloride (185 MBq) after conjugation with freshly prepared ccDTPA. Conjugated-rituximab was obtained by the addition of 1 ml of a rituximab pharmaceutical solution (5 mg/ml, in phosphate buffer, pH = 7.8) to a glass tube precoated with freshly prepared ccDTPA (0.01–0.1 mg) at 25°C. Sm-153 chloride was obtained by a thermal neutron flux (5×10^{13} n·cm^{-2}·s^{-1}) of an enriched $^{152}Sm_2O_3$ sample, dissolved in acidic media. Radiolabeling was performed in one hour by the addition of DTPA-rituximab conjugate at room temperature. Radiochemical purity of 96% (ITLC) and 98% (HPLC) were obtained for the final radioimmunoconjugate (specific activity = 120 TBq/mmol). The final isotonic ^{153}Sm-rituximab complex was checked by gel electrophoresis for protein integrity retention. Biodistribution studies in normal rats were performed to determine radioimmunoconjugate distribution up to 24 h. SPECT images were also obtained using 103 keV photons up to 48 h.

Key words: radiopharmaceutical • samarium-153 • rituximab • targeted therapy • SPECT

A. Bahrami-Samani
Faculty of Nuclear Engineering and Physics,
Amirkabir University of Technology,
P. O. Box 15875-4413, Tehran, Iran

M. Ghannadi-Maragheh,
Agricultural, Medical and Industrial Research School (AMIRS),
P. O. Box 31485-498, Karaj, Iran
and Faculty of Nuclear Engineering and Physics,
Amirkabir University of Technology,
P. O. Box 15875-4413, Tehran, Iran

A. R. Jalilian✉, H. Yousefnia, J. Garousi, S. Moradkhani
Radiopharmaceutical Research & Development Laboratory (RRDL),
Nuclear Science and Technology Research Institute (NSTRI),
14395-836 Tehran, Iran,
Tel./Fax: +98 261 443 6397,
E-mail: ajalilian@nrcam.org

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Introduction

The importance of existence and application of radiolabeled anti-CD20 monoclonal antibodies at non-myeloablative doses in treating B-cell NHL is well recognized throughout the world. Several agents such as ^{131}I-tositumomab (Bexxar) and ^{90}Y-ibritumomab tiuxetan (Zevalin) have been already applied in clinics with therapeutic response rates of 25 to 40% with a median response [3, 12, 13, 21].

However, I-131 labeled antibody suffers from radiolysis and instability in formulations, increasing patient absorbed dose and thyroid defects. On the other hand, Y-90 is not available for most of the world countries due to low availability, high expenses and import restrictions. The development and application of other beta-emitters labeled anti-CD20 immunoconjugates is undergoing in many parts of the world, due to the availability of research reactors. Many beta-emitters such as Sm-153, Lu-177 and Ho-166 can be produced in reasonable amounts using (n,γ) reactions.

Samarium-153 is of the most important therapeutic nuclides due to low energy beta particles interesting for

targeted therapy modalities. ^{153}Sm ($T_{1/2}$ = 46.7 h) is usually prepared by neutron irradiation of natural and/or enriched $^{152}Sm_2O_3$.

Many studies have focused on the development of anti-CD20 radioimmunoconjugates for the study of antibody metabolism, CD20 antigen localization and therapeutic response determination [2, 6].

Various monoclonal antibodies have already been labeled using Sm-153 for radioimmunotherapy using DTPA-conjugates, such as antiCEA [4] and anti-CD33 [18] for radioimmunotherapy.

It is already known that the cation $^{153}Sm^{3+}$ has good chelating properties with polyaminopolycarboxylic acids, like DTPA [1], thus in this work we chose DTPA as the intermediate chelating agent, which can be linked to the antibodies via the bicyclic anhydride (ccDTPA).

We have recently reported the production of radiolabeled rituximab radioimmunoconjugates mostly used in the imaging studies of lymphoma-B models using cyclotron-derived radionuclides such as Ga-67 [10] and In-111 [11] using DTPA and DOTA intermediates. Following development of the radiolabeling and quality control methods under sterile conditions in our labs, we have now focused on the production of beta-emitter labeled rituximab conjugate for ultimate radioimmunotherapy applications in the country.

Experimental

Materials

Production of ^{153}Sm was performed at the Tehran Research Reactor (TRR) using ^{152}Sm (n,γ) ^{153}Sm nuclear reaction. Samarium-152 with a purity of >98% was obtained from ISOTEC Inc. Sephadex G-50, sodium acetate, phosphate buffer components and methanol were purchased from Sigma-Aldrich Chemical Co. UK. Rituximab (MabThera™) was a pharmaceutical sample purchased from Roche Co., USA, and was used without further purification. Cyclic DTPA dianhydride was freshly synthesized in our laboratory based on the conventional method [8].

Radio-chromatography was performed by counting Whatman no. 2, using a thin layer chromatography scanner, Bioscan AR2000, Paris, France. 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 Whatman Partisphere C-18 column 250 × 4.6 mm, Whatman Co. NJ, USA. Calculations were based on the 103 keV peak from ^{153}Sm. 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. All of the rats were Male NMRI purchased from Pasteur Institute of Iran, weighing 180–200 g, in each group/interval 5 rats were used and kept at routine day/night light program and under common rodent diet pellets.

Procedures

Production and quality control of $^{153}SmCl_3$ solution

Samarium-153 was produced by neutron irradiation of 100 µg of enriched $^{152}Sm_2O_3$ (^{152}Sm, 98.7% from ISOTEC Inc.) according to reported procedures [5] in the Tehran Research Reactor at a thermal neutron flux of 5×10^{13} n·cm^{-2}·s^{-1} for 5 d. Specific activity of the produced ^{153}Sm was 750 mCi/mg. The irradiated target was dissolved in 200 µl of 1.0 M HCl, to prepare $^{153}SmCl_3$ and diluted to the appropriate volume with ultra pure water, to produce a stock solution. The mixture was filtered through a 0.22 µm biological filter and sent for use in the radiolabeling step. The radionuclidic purity of the solution was tested for the presence of other radionuclides using beta spectroscopy as well as HPGe spectroscopy for the detection of various interfering beta and gamma emitting radionuclides. The radiochemical purity of the $^{153}SmCl_3$ was checked using 2 solvent systems for ITLC (A: 10 mM DTPA pH 4 and B: ammonium acetate 10%:methanol (1:1)).

Conjugation of cyclic DTPA di-anhydride with rituximab

The chelator diethylenetriamine penta-acetic acid dianhydride was conjugated with rituximab using a small modification of the well known cyclic anhydride method. 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 rituximab (5 mg, 0.5 ml, pH 7.5) 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 a 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 ^{153}Sm

The antibody conjugate was labeled using an optimization protocol according to the literature [15]. Typically, 40–400 MBq of ^{153}Sm-chloride (in 0.2 M HCl) was added to a conical vial and dried under a flow of nitrogen. To the above vial acetate buffer (200 µl, pH 4.8) was added and the vial vortexed for 30 s. The protein containing fraction with the maximum protein content was added in 1 ml of phosphate buffer (0.1 M, pH = 8) to the vial and mixed gently for 30 s. The resulting solution was incubated at 25°C for 1 h. Following incubation, ITLC and HPLC were performed to determine the radiochemical purity and in the case of low radiochemical purity the radiolabeled antibody conjugate was purified from free ^{153}Sm by size exclusion chromatography 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 a freshly prepared Folin-Colciteau® reagent (5 µl prepared by mixing 25 µl of fresh CuTartrate solution) and 10 µl of the eluted fractions. The fractions containing the proteins (visible blue color by naked eye) with the maximum radioactivity was tested for purity by ITLC using a radio-TLC scanner. Control labeling experiments were also performed using $^{153}SmCl_3$, and DTPA with $^{153}SmCl_3$.

Preparation of ^{153}Sm-DTPA for control studies

For control experiments, ^{153}Sm-DTPA was prepared for R_f and retention time studies in chromatographic methods. For preparation, 37 MBq of ^{153}Sm-chloride (in 0.2 M HCl) was added to a conical vial and dried under a flow of nitrogen. To the Sm-containing vial was added phosphate buffer (0.1 M, 200 µl, pH 8) and the vial vortexed for 30 s (final pH 7.5). A solution of DTPA (2.5 mM) in 300 µl of phosphate buffer (0.1 M, pH = 8) was added to the first vial and mixed gently for 30 s. The resulting solution was incubated at 25°C for 1 h.

Quality control of ^{153}Sm-DTPA-rituximab

Thin-layer chromatography. From the final product, 5 µl was applied to a Whatman no. 2 paper strip followed by developing in 10 mM DTPA (pH 3). Radioactivity was determined by a RTLC scanner.

High performance liquid chromatography. HPLC was performed on the final preparation using an 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 antibody 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 $^{153}Sm^{3+}$ cation can be complexed with citrate anion, however pre-complexed Sm-DTPA-rituximab was not challenged with the addition of citrate chelate [7].

SDS-polyacrylamide gel electrophoresis

The radioimmunoconjugate was analyzed for integrity by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The radiolabeled mAb was evaluated with and without reduction by 2-mercaptoethanol. Approximately 200,000 cpm of each preparation was applied per lane and the 4–20% polyacrylamide were run according to the method of Laemmli [14].

Stability testing of the radiolabeled compound in final formulation

Stability of ^{153}Sm-DTPA-rituximab in PBS was determined by storing the final solution at 4°C for 2 d and performing frequent ITLC analysis to determine radiochemical purity. The stability of the conjugated DTPA-rituximab stored at –20°C for more than 3 months was also investigated. ITLC analysis of the conjugated product was performed to monitor degradation products or other impurities. After subsequent ^{153}Sm-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

Radiolabel stability was assessed by size exclusion chromatography 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; 1 mL fractions were collected.

Biodistribution of ^{153}Sm-radioimmunoconjugates to normal rats

To determine its biodistribution, ^{153}Sm-DTPA-rituximab was administered to normal rats. A volume (50–100 µl, 1–2 mg/ml of the antibody determined by UV spectrophotometric method) of final ^{153}Sm-DTPA-rituximab solution containing 180±5 µCi radioactivity was injected intravenously to rats through their tail vein. The animals were sacrificed at the exact time intervals (1, 2 and 24 h), and the specific activity of different organs was calculated as percentage of injected dose per gram using an HPGe detector.

SPECT imaging of ^{153}Sm-DTPA-rituximab in normal rats

Images were taken at 1, 24 and 48 h after administration of the radiopharmaceutical by a dual-head SPECT system. The mouse-to-high energy septa distance was 12 cm. The useful field of view (UFOV) was 540 × 400 mm. The spatial resolution was 10 mm FWHM at the CFOV. 64 projections were acquired for 30 s per view with a 64 × 64 matrix.

Results

The radionuclide was prepared in a research reactor according to regular methods with a range of specific activity 600–750 mCi/mg for radiolabeling use, after counting the samples on an HPGe detector for 5 h very slight amount of impurities were recorded (Fig. 1). The spectrum showed that the impurities are majorly Eu radionuclides (Table 1).

The radioisotope was dissolved in acidic media as a starting sample and was further diluted and evaporated for obtaining the desired pH and volume followed by sterile filtering.

The conjugated DTPA-rituximab fractions containing the maximum protein content were labeled with ^{153}Sm-$SmCl_3$ solution. The samples were checked by ITLC to find the best time scale for labeling. After an hour, the free ^{153}Sm/conjugated ^{153}Sm ratio in the labeled sample remained unchanged at 4:96. The mixture could then be passed through another Sephadex G-50 size exclusion chromatography column in order to remove unbound ^{153}Sm cation and/or other low molecular weight impurities.

The eluted fractions were checked by Folin-Colciteau® reagent and for the presence of radioactivity

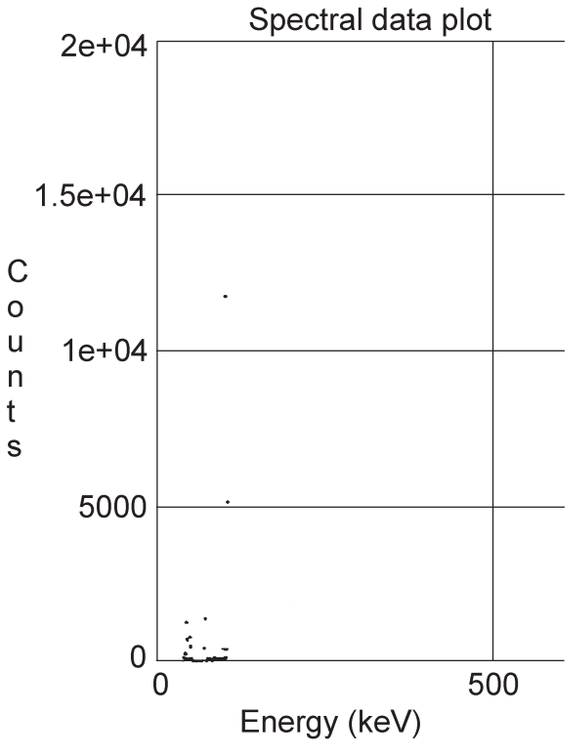

Fig. 1. Gamma spectrum for Sm-153 prepared by neutron irradiation of Sm-152 sample using an HPGe detector.

in order to determine the ^{153}Sm-DTPA-rituximab containing fractions. Fraction with the highest radioactivity which contained the maximum color absorbance was chosen as the suitable final product with appropriate specific activity for animal tests. Instant thin layer chromatography using various mobile and stationary phases was performed in order to ensure the existence of only the desired radiolabeled antibody. In all ITLC tests, radiolabeled antibody stayed at the origin while other species migrated to other R_fs, depending on the mobile phase used. For Sm^{3+} detection, the best eluent system was 10 mM DTPA aqueous solution in R_f of 0.8. For Sm-DTPA detection, ammonium acetate 10%:methanol (1:1) can be used separately to ensure the absence of any free DTPA in the purified conjugated antibody. Due to the size and charge of the protein (≈ 150,000 D), ^{153}Sm-DTPA-rituximab remains at the origin in all the systems used (Fig. 2).

Table 1. The radionuclidic impurities and their percentages in the final Sm-153 samples produced from enriched Sm-152 (n = 5)

Radionuclides	Impurity (%)
Eu-154	< 2.27e-4
Eu-155	< 1.02e-4
Eu-156	< 4.90e-4

Using ITLC tests in several radiolabeling runs, the labeling efficiencies and specific activities of the final samples were determined (Table 2).

In HPLC studies, reverse phase column in a gradient buffer system as eluent was used. The fast eluting component (1.92 min) was shown to be a mixture of free ^{153}Sm and ^{153}SmDTPA. Both compounds are ionic, so they are eluted at the same retention time. The radiolabeled protein was finally washed out at 28.15 min (Fig. 3).

Due to the UV absorbance of DTPA moiety, the UV detector chromatogram cannot be used for chemical purity determination, however using free DTPA and Sm-153 for control studies, it was shown that 98% of present DTPA at the reaction pH will form ^{153}Sm-DTPA species which can be analyzed by a flow scintillation

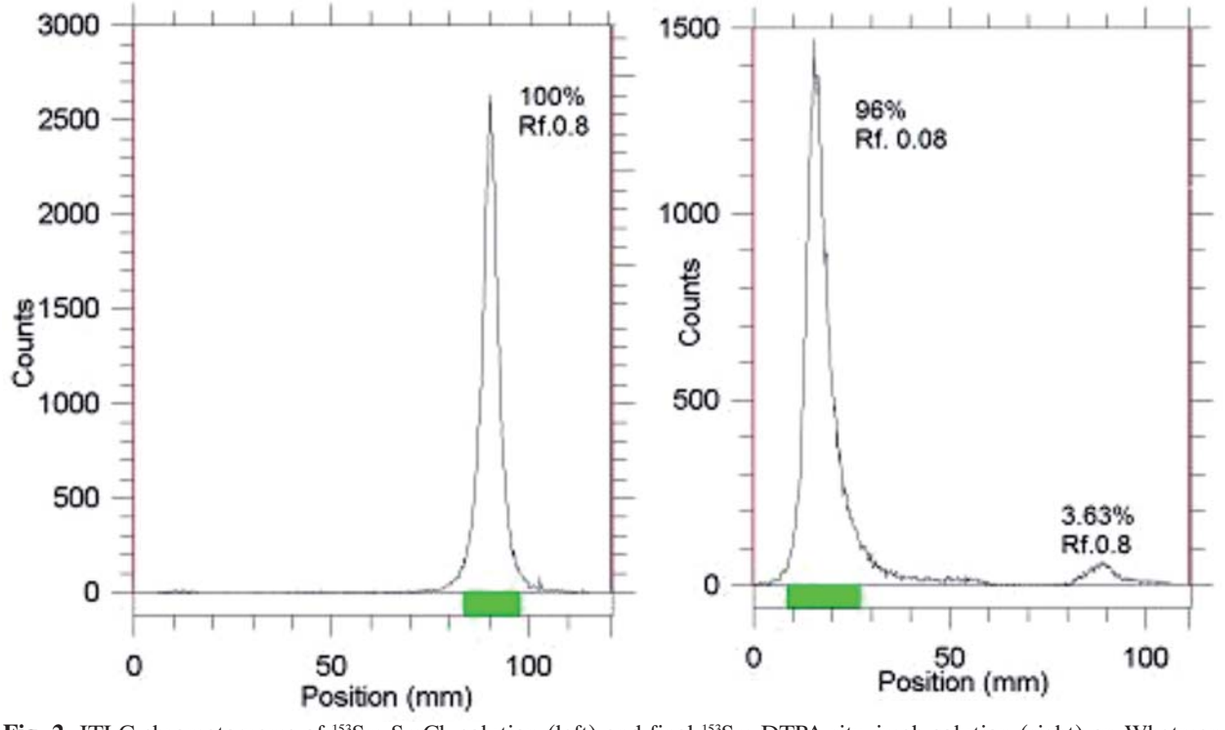

Fig. 2. ITLC chromatograms of ^{153}Sm-$SmCl_3$ solution (left) and final ^{153}Sm-DTPA-rituximab solution (right) on Whatman no. 2 paper using 10 mM DTPA solution (pH 3).

Table 2. The labeling efficiencies and specific activities of the prepared compounds in various runs

Irradiation times (days)	2	3	4	5
Labeling efficiency (%)	52±4	57±5	65±8	78±6
Specific activities (TBq/mM)	498	550	390	580
Radiochemical purities (%)	95±2	97±1	96±2	98±1

Table 3. Radiochemical purity of final labeled compound in final solutions and in presence of human serum at 37°C

Time	Radiochemical purity in final solution (%)	Radiochemical purity in presence of human serum (%)
2 h	98±2	97±2
4 h	96±1.5	94±1
24 h	89±3	80±2
48 h	78±2	57±6

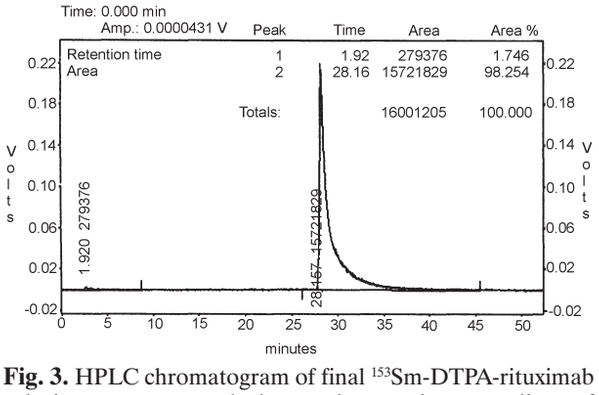

Fig. 3. HPLC chromatogram of final ^{153}Sm-DTPA-rituximab solution on a reversed phase column using a gradient of acetate/citrate buffer.

analyzer. Assuming the only impurity in the whole process would be DTPA.

Since radiolabeling of each antibody molecule with one radioisotope atom at optimal theoretical conditions can be satisfactory, we chose the 1:1 molar ratio. The only possible way to check the yield was RTLC/HPLC analysis of final radiolabeled antibody at various molar ratios.

After incubation of ^{153}Sm-DTPA-rituximab with freshly prepared human serum at described conditions in the methods, 96–98% of the radioactivity eluted at the same fraction as ^{153}Sm-DTPA-rituximab, using size exclusion chromatography (Table 3). Thus, there was no evidence for either degradation or transchelation of ^{153}Sm to other serum proteins over a time period consistent with the normal blood clearance time of rituximab.

In order to demonstrate the integrity of the protein after conjugation and radiolabeling gel electrophoresis was performed on the SDS PAGE gels using 16% bisacrylamide gel. The loaded samples were a commercial rituximab sample, DTPA-rituximab and radiolabeled protein samples a week after the experiment while kept in the fridge. The 3 samples showed similar pattern of migration in the gel electrophoresis.

Interestingly, the SDS PAGE results were checked with a reported commercial rituximab sample. Figure 4 shows the SDS-PAGE patterns for DTPA conjugate in contrast to starting rituximab sample and radiolabeled immunoconjugates.

The animals were sacrificed by ether asphyxiation at selected times after injection (1, 2 and 24 h). Dissection began by drawing blood from the aorta, followed by collecting blood, heart, spleen, muscle, brain, bone, kidneys, liver, intestine, stomach, lung and skin samples. The tissue uptakes were calculated as the percent of area under the curve of the related photo peak per gram of tissue (% ID/g) (Table 4).

At 1–2 h post injection, the activity is mainly removed from the blood which is in agreement with the other reported labeled antibodies [19]. Likewise other radiolabeled proteins, the labeled antibody is accumulated in the liver.

The brain did not show any significant uptake over the period of time. This had already been shown by ^{123}I-antiCD20 conjugate biodistribution studies [2]. High uptake in spleen and reticuloendothelial organs was observed, which is due to the final accumulation of B-lymphocytes carrying the radioimmunoconjugate on their surface. The increasing uptake of spleen as a course

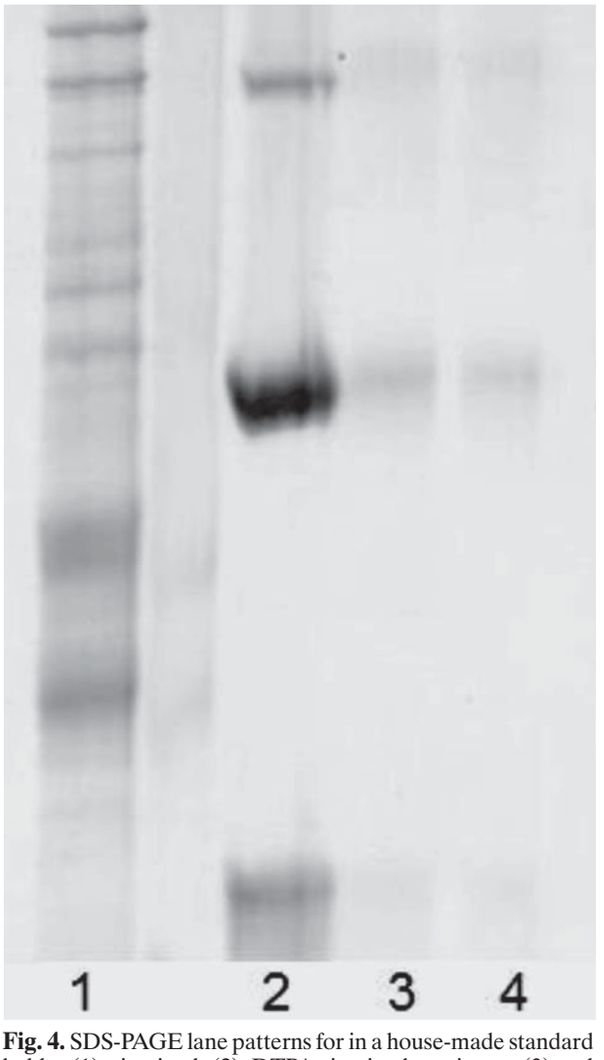

Fig. 4. SDS-PAGE lane patterns for in a house-made standard ladder (1), rituximab (2), DTPA-rituximab conjugate (3) and ^{153}Sm-DTPA-rituximab conjugate (4).

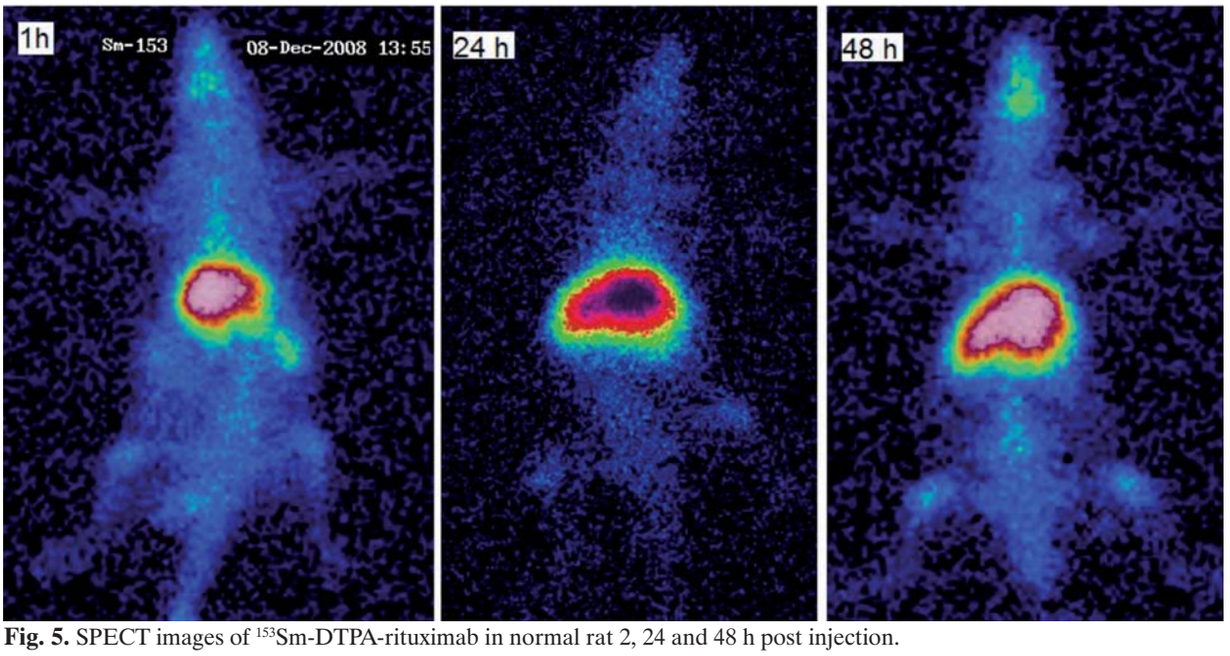

Fig. 5. SPECT images of ^{153}Sm-DTPA-rituximab in normal rat 2, 24 and 48 h post injection.

Table 4. Percentage of injected dose per gram (ID/g %) of ^{153}Sm-DTPA-rituximab in normal rat tissues at 1, 2 and 24 h post injection

Tissues	1 h	2 h	24 h
Blood	0.050	0.037	0.019
Heart	0.078	0.042	0.115
Liver	12.185	8.558	14.990
Kidney	0.503	0.500	1.439
Skin	0.058	0.021	0.042
Muscle	0.019	0.012	0.008
Bone	0.332	0.219	0.578
Intestine	0.054	0.037	0.058
Lung	7.844	7.143	0.146
Spleen	8.408	2.713	6.025
Brain	0.005	0.002	0.078
Stomach	0.078	0.030	0.066

of time is a direct result of the depletion of circulating B-cells occurring rapidly after administration to the mammals. This has been already shown in human, which is an important sign of therapy in lymphoma patients [9].

As a natural reaction to the depletion of the lymphocytes, the reticuloendothelial system including spleen will be the final possible reservoir of the depleted lymphocytes. However, a direct resemblance of CD-20 antigen in human and rats has not been demonstrated. On the other hand, rituximab natural binding was found on lymphoid cells in the thymus, the white pulp of the spleen, and a majority of B-lymphocytes in peripheral blood and the lymph nodes in human being [16], this has been observed in our studies on the normal rats as well.

Significant accumulation in the lungs was also observed. Interestingly, we found reports of severe pulmonary reactions using anti-CD20 with pulmonary infiltrates or edema in human. Acute symptoms appear within 1–2 h of the initiation of the 1st infusion [17]. However, it usually fades after 18–24 h which can be observed in Table 4. Slight bone uptake can be attributed to the presence/formation of Sm-153 cation released in blood leading to kidney excretion especially at 24 h.

The best time period for scanning showed to be 24 h post injection. Up to 2 h post injection a rather high accumulation was observed in the liver of the rats, however, a dispersed radioactivity was detectable in the whole body. At 24 h the activity is concentrated in the liver and the background is negligible (Fig. 5). After 48 h the re-absorption of liver metabolites through enterohepatic cycle can happen through the intestine, resulting in an increase of the whole-body background. Interestingly, this data is comparable to other reported methods using various radionuclides to radiolabeled antibodies with the maximum uptake in liver [20].

Conclusions

For [^{153}Sm]-DTPA-rituximab, the radiochemical purity was 98% and the labeling and quality control took 1 h. The radiolabeled complex was stable in human serum for at least 24 h and no significant amount of free ^{153}Sm as well as ^{153}Sm-DTPA was observed. The final preparation was administered to normal rats and biodistribution of the radiopharmaceutical was checked 1–48 h later. ^{153}Sm-rituximab is a potential probe for therapy of lymphomas. The experiments on lymphoma animal models should be performed for this radioimmunoconjugate before human use.

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