NUKLEONIKA 2005;50(3):91–96

ORIGINAL PAPER

Development of ^{111}In-DTPA-human polyclonal antibody complex for long-term inflammation/infection detection

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Abstract Human polyclonal antibody (HIgG) was successively labeled with ^{111}In-indium chloride after residulation with freshly prepared cyclic DTPA-dianhydride. The best results of the conjugation were obtained by the addition of solid DTPA-dianhydride (0.1–0.3 mg) to 100 μl of the HIgG solution (0.2–0.4 mg/ml) at pH = 6 in phosphate buffer media at 25°C with continuous stirring for 30 min. Radio-thin-layer chromatography showed an overall radiochemical yield of 96–99% at optimized conditions (specific activity = 300–500 MBq/mg, radiochemical purity >98%). The final isotonic ^{111}In-DTPA-HIgG complex was checked by radio-TLC to ensure the formation of only one species followed by filtration through a 0.22 μ filter. Preliminary long-term *in vivo* studies in turpentine-oil induced inflammation in rat model was performed to determine late complex distribution of the radioimmunoconjugate. The target/skin and target/blood ratios were 27 and 51 after 24 h, and 23 and 51 after 110 h, showing a high selectivity of the radiopharmaceutical for inflammatory lesions.

Key words radiopharmaceuticals • indium-111 • human polyclonal antibody • inflammation • turpentine oil

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Received: 7 March 2005
Accepted: 19 May 2005

Introduction

Radiolabeled immunoglobulins have been prepared using various single photon emission computed tomography (SPECT) radioisotopes such as In-111 [12], Tc-99m [11], etc., out of which the only FDA approved radioimmunoconjugates are those containing In-111 radionuclide. ^{111}In-Ritauximab (Zevalin™) was approved by FDA for detection of lymphosarcoma and B cell lymphomas in 2002 [8]. ProstaScint, Mouse IgGK1, 7E11-C5.3 antibody against PSA, conjugated with diethylene-triamine-pentaacetate (DTPA), has been successfully used in detection of prostate cancer [7]. Finally, OncoScint, another DTPA conjugated CYT-103 antibody is a valuable tool in the detection of colorectal and ovarian tumors in clinic [9].

^{111}In-labeled HIgG has been extensively tested in a large number of clinical studies. It has shown excellent performance in the localization of musculoskeletal infection and inflammation [16]. In addition, good results have been reported in pulmonary infection, particularly in immunocompromised patients [1, 17], and abdominal inflammation [13].

In this study, a precise labeling strategy was employed using freshly-prepared DTPA cyclic dianhydride, with various HIgG concentrations. All experiments were checked by radiochemical tests and their immunoreac-

tivity against inflammatory lesions in rats. Finally, an optimized radiolabeling method for developing a highly reactive diagnostic tool for inflammation using 3.7×10^6 Bq ^{111}In-DTPA-HIgG in experimental animals using a long term protocol study has been introduced for the first time.

Experimental

Materials

Sephadex G-50 was purchased from Pharmacia, Italy. DTPA (acid form), 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. ^{1}H-NMR spectra were obtained on a FT-80 (80 MHz) 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) multichannel analyzer. Calculations were based on the 171 keV peak from ^{111}In. All values were expressed as mean ± standard deviation (Mean ± SD) and the data were compared using student T-test. Statistical significance was defined as $P < 0.05$.

Methods

Electroplating of the natural Cd targets

Cadmium electroplating over a copper surface was performed according to the conditions given in the literature [15]. Briefly, a mixture of $3CdSO_4 \cdot 8H_2O$, KCN, Brij and hydrazine hydrate with a final volume of 450 ml DDH_2O (pH 13) was used as the electroplating bath for cadmium deposition on a copper backing (constant current: 320 mA, stirring rate 780 rpm, time: 0.5 h). After the deposition of about 500 mg cadmium layer, the targets were wrapped in Parafilm® coatings to avoid atmospheric oxygen exposure. Finally, the target was sent for irradiation.

Preparation of ^{111}In-$InCl_3$ solution

^{111}In-indium chloride was prepared by 20 MeV proton bombardment of the natural Cd-electroplated copper backing prescribed above with a high purity (>95%) in a 30 MeV cyclotron for 48 min and 80 μAh and an intensity of 100 μA. After dissolution of the irradiated target by conc. HBr, the solution was passed through a cation exchange Dowex 50 × 8 resin, pre-conditioned by 20 ml of conc. HBr. The resin was then washed by an HBr conc. solution (3 × 25 ml). In order to remove the unwanted impurities of Cd and Cu, the resin was totally washed with DDH_2O (3 × 25 ml). Indium-111 was eluted with HCl 1 M (25 ml) as $^{111}InCl_3$ for labeling use.

- Radionuclide purity. Gamma spectroscopy of the final sample was carried out by an HPGe detector.
- Chemical purity. The presence of copper and cadmium cations were checked by colorimetric assays. The formation of colored metal complexes was determined by standard sample concentrations. Cu^{2+} was an important undesired ion present in target support. Dithizone is the most sensitive reagent for the determination of Cu^{2+} ions. In an acidic medium containing an excess of dithizone, copper(II) forms the violet dithizonate ($Cu(HDz)_2$) [14].

The target material was cadmium and its presence was checked using a colorimetric assay using a dithizone reagent in the presence of alkaline dimethylglyoxime and NaK tartrate according to the literature [14] (Table 1).

Preparation of fresh cyclic DTPA dianhydride for optimal protein residulation

This compound was prepared according to methods previously given in the literature with slight modifications [3]. Briefly, DTPA in the 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 finally with anhydrous ether. 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 are presented in Figs. 2 and 3.

Conjugation of cyclic DTPA dianhydride with the human immunoglobulin (HIgG)

A solution of HIgG, was prepared (0.2 mg/ml in PBS). A solid portion of cyclic DTPA dianhydride (0.1–0.2 mg) was added to a portion of the HIgG solution (0.4 ml)

Table 1. Cations determined by colorimetric assay in this work [14]

Cation	Chelate	USP limit	EP limit	Absorbance	Source
Copper	dithizone	6	5	yellow	backing
Iron	di-pyridyl	5	5	pink	valves
Cadmium	dithizone/DMG	5	8	blue	target

[1] United States Pharmacopeiae; [2] European Pharmacopeiae.

and mixed slowly in a shaker at room temperature for 10, 20, 30, 45 and 60 min. Each conjugation mixture was then passed through a Sephadex G-50 column (2 × 10 cm) 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 immunoconjugate were combined. The final concentration of the conjugated antibody was between 0.1–0.15 mg/ml. This fraction was kept at 4°C and was used for radiolabeling step.

Radiolabeling of the antibody conjugate with ^{111}In

The antibody conjugate was labeled using an optimization protocol according to the literature [6]. Typically, the conjugated fraction (35 μg) in 100–300 μl of sodium acetate buffer (0.1 M, pH 7.2) was mixed with 18.5–22.2 MBq of ^{111}In-acetate, which was prepared by adding 250 μl of citric acid (0.1 M, pH 1.7) and 100 ml of 0.1 M sodium acetate to 40 μl of $InCl_3$. The resulting solution had a final pH of 5.1 and was incubated at room temperature for 30 min. Following incubation, the radiolabeled antibody conjugates were purified from free ^{111}In by gel filtration on the Sephadex G-50 column (10 ml bed volume) and eluted with PBS. Fractions (0.5 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 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 $^{111}InCl_3$ in acetate/citric acid, and DTPA with $^{111}InCl_3$. Both reaction mixtures were passed through separate gel filtration columns and eluted with PBS.

Quality control of ^{111}In-DTPA-polyclonal human immunoglobulin

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 Rf_s of free In^{3+} and ^{111}In-DTPA 0.5 and 0.9, respectively, while radiolabeled protein stays at the bottom ($R_f = 0.0$).

Stability of ^{111}In-immunoconjugate complex in serum

A sample of ^{111}In-conjugate (3.7×10^3 – 1.85×10^5 Bq) was added to a shaken human serum mixture (450 ml) in a 37°C incubator under a nitrogen atmosphere. A micropipet sample (5 μL) was taken from the shaken mixture every 30 min and spotted on a silica gel paper and developed in a mixture of ammonium acetate (10%):methanol (9:1) as the mobile phase. Free In^{3+} and ^{111}In-DTPA-immunoconjugate migrated to the Rf_s of 0.5 and 0.9, respectively.

Biodistribution of ^{111}In-DTPA-HIgG to normal rats

To determine its biodistribution, ^{111}In-DTPA-HIgG was administered to normal rats. A volume (50–100 μl) of final ^{111}In-DTPA-HIgG solution containing $5.55\times10^6 \pm 1.85\times10^5$ Bq radioactivity was injected intravenously to rats through their tail vein. The animals were sacrificed at exact time intervals (24 and 110 h), and the specific activity of different organs was calculated as percentage of injected dose using a radiometer.

Biodistribution of ^{111}In-DTPA-HIgG to inflammatory-lesion bearing rats

Animal studies and procedures were performed using previous methods for evaluation of inflammation seeking compounds. Turpentine oil (40 μl) was injected SC to the dorsal area of normal rats weighing 150–200 g. After 6–8 days sample animals were separated and kept for the distribution studies. The distribution of ^{111}In-DTPA-HIgG among tissues was determined for untreated rats and for rats with inflammatory lesions. A volume of final ^{111}In-DTPA-HIgG solution (0.1 ml) containing an activity of 4.4 MBq (≤ 15 mg in 100 μL) was injected to animals *via* their 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 fixed geometry.

The animals were sacrificed by ether anesthesia at selected times after injection (24 h and 110 h), the tissues (blood, heart, lung, spleen, intestine, foeces, skin, bladder, kidneys, liver, muscle and bone) were weighed and rinsed with normal saline and their specific activities were determined with an HPGe detector equipped with a sample holder device as percent of injected dose per gram of tissues.

Results and discussion

Radiolabeled human polyclonal IgG localizes at focal sites of infection/inflammation. ^{111}In-IgG scintigraphy significantly contributed to the diagnostic process in patients with fever of unknown origin [2] and the detection of focal infection [20]. Indium-111-IgG scintigraphy is a sensitive tool for the detection of infectious bone and joint disease. Recently, ^{111}In-HIgG has been used in clinical studies of antibody distribution and pharmacokinetics [18, 19]. Initially it was hypothesized that HIgG was retained in inflammatory foci due to the interaction with Fc-g receptors as expressed on infiltrating leukocytes [5]. Later studies showed that radiolabeled HIgG accumulates in infectious foci by nonspecific extravasations due to the locally enhanced vascular permeability as well [4]. Thus, ^{111}InHIgG can be used for sensitive detection of inflammation/infection foci.

There have been many low-dose InHIgG administrations followed by 24 h tissue dose determination studies in the literature. In this study we used a rather high-dose InHIgG administration in animals with a rather long-term data acquisition showing a very good distribution pattern after 110 h.

Production of ^{111}In-indium chloride

Irradiation of the natural electroplated cadmium on a copper backing, produced a large amount of ^{111}In in the form of chloride after elution using 0.05 M HCl. Gamma spectroscopy of the final sample showed a radionuclide purity higher than 97% showing the presence of 171 and 245 keV gamma energies. The chemical purity of the final solution was checked by colorimetric assays for Cd, Cu cations that both were under the permitted limits as shown in Table 1.

Preparation and structure confirmation of DTPA cyclic dianhydride

In order to prepare the bi-functional ligand, DTPA cyclic dianhydride, which was not cost effective, we tried the general procedure for its preparation [6]. The reaction was performed in pyridine containing DTPA acid form and acetic anhydride. The filtered mass was washed with cold acetic anhydride to remove the 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 (Fig. 1). Washing/drying steps were very important. 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.

DTPA cyclic anhydride was characterized by IR spectroscopy. The formation of 1730 cm^{-1} peak indicated anhydride carbonyl group formation which is accompanied by a weaker 1695 cm^{-1} carboxylic acid peak of the untouched COOH. The IR spectrum of cyclic DTPA-dianhydride is shown in Fig. 2.

^{1}H NMR spectrum of the above compound was recorded in DMSO at 25°C. The chemical shifts of CH_2CO groups have the lowest field are very close so

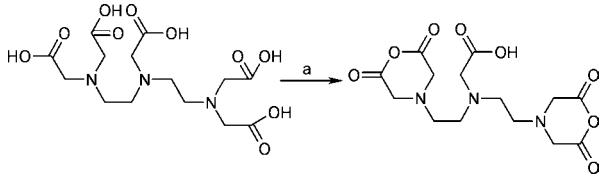

Fig. 1. Schematic diagram of the synthesis of DTPA cyclic dianhydride.

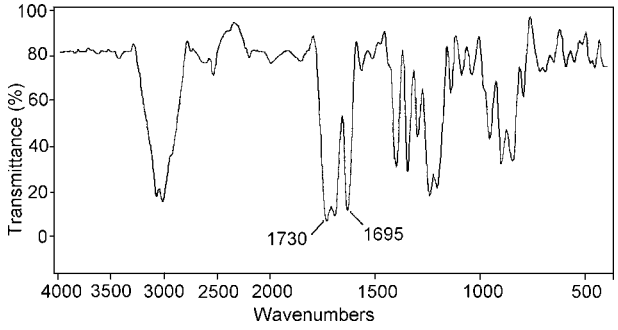

Fig. 2. FT-IR spectrum of DTPA cyclic dianhydride prepared in this study.

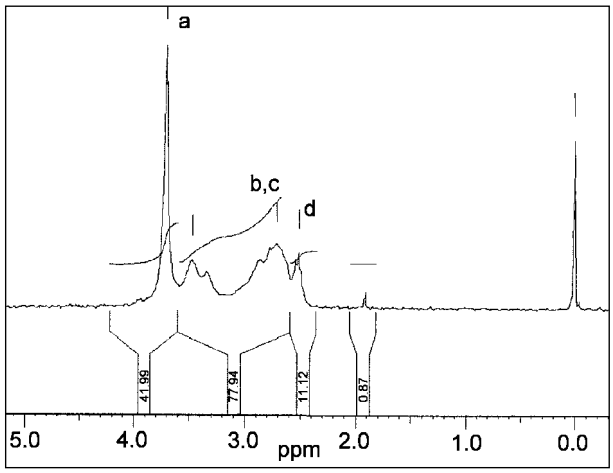

Fig. 3. ^{1}H NMR spectrum of DTPA cyclic dianhydride prepared in this study.

that a major singlet is observed around 3.76 ppm (a). The NCH_2CH_2N groups are more shielded and because of their similarity, a broad multiplet is observed at 2.6–2.56 ppm (b, c). The DMSO peak is observed at 2.5 ppm as a multiplet (d). The ^{1}H NMR spectrum of cyclic DTPA-dianhydride is given in Fig. 3.

Conjugation of the HIgG with DTPA cyclic dianhydride and radiolabeling of HIgG with ^{111}In

The labeling yield of ^{111}In-DTPA-HIgG has been studied in a wide range of antibody/DTPA ratios in order to optimize the process and to improve ^{111}InDTPA-HIgG performance *in vitro* (Table 2). The overall radiolabeling efficiency was over 77% and the specific activity was kept in the range of 300–500 MBq/mg. The reagent ratios against radiolabeling efficiency in our experiments are demonstrated in Table 2.

The conjugated HIgG fractions containing the maximum protein content were mixed with ^{111}In-$InCl_3$ 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 ^{111}In/conjugated ^{111}In 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 ^{111}In cation.

Table 2. Percentage radiolabeling efficiency of antibody conjugate

Antibody/DTPA molar ratio	Radiolabeling efficiency (%)
1:100	89.0
1:50	90.3
1:20	89.3
1:10	85.8
1:2	81.1
1:1	75.6

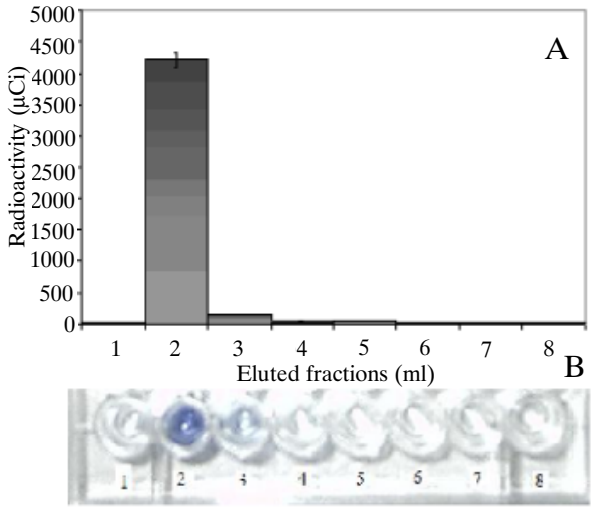

Fig. 4. Radioactivity (A) and colorimetric (B) assays of ^{111}In-DTPA-HIgG fractions eluted from a gel filtration column.

The eluted fractions were checked by Folin-Colciteau® reagent and for the presence of radioactivity in order to determine the ^{111}In-DTPA-HIgG containing fractions. Figure 4 shows the radioactivity content of the eluted fractions as well as the protein presence. Fraction number 2 was chosen as the suitable final product with appropriate specific activity for animal tests.

In vitro serum stability

Radiolabeled conjugate was examined for stability in mouse serum over a 48 h incubation period at 37°C. Approximately 95% of the activity was present at $R_f = 0$ as was expected for the labeled protein in RTLC (NH_4OAc:MeOH; 9:1) and no significant release of free ^{111}In was detected over this time period. This observation provides evidence for similarities in radiolabeling efficiency and serum stability of other reported radiolabeled antibody conjugates [10].

Induction of inflammation in normal rats

Inflammation-bearing rats: turpentine oil (40 μl) was injected SC to the dorsal area of 5 groups of rats weighing 150–175 grams. After 7 days sample animals were ready

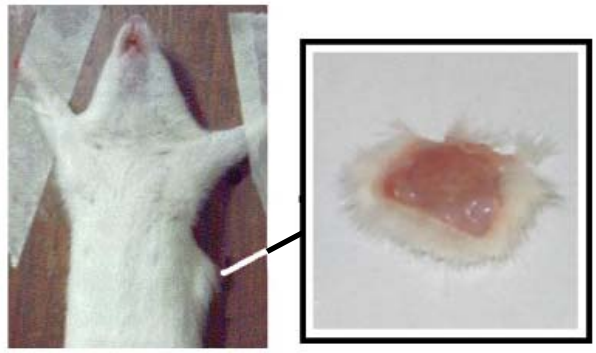

Fig. 5. The size and shape of inflammatory lesions in the animal and after dissection.

for biodistribution studies while inflammatory tissues weighed to suitable amounts (0.7 ± 0.05 g) (Fig. 5).

Biodistribution studies

The distribution of [^{111}In]$InCl_3$ and [^{111}In]HIgG among tissues were determined for untreated rats and for rats with inflammatory lesions. A volume (0.1 ml) of the final [^{111}In]HIgG solution containing 4.4–5.2 MBq radioactivity (≤ 6 mg 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 (1 and 4 days), the tissues (blood, heart, spleen, kidneys, liver, intestine, stomach, lung, skin and inflamed tissue) and foeces were weighed and their specific activities were determined with a γ-ray scintillation as a percent of the injected dose per gram of tissues (Figs. 6 and 7).

As can be concluded from Fig. 6, after 24 h the maximum activity is present in the kidney which is possibly due to excretion of ^{111}In after complex dissociation in the liver and a rather nice accumulation of radioimmunoconjugate in the inflamed tissue. Lower accumulations have been observed in the gastrointestinal system due to entero-hepatic excretion. After 110 h, the background radioactivity from the gastrointestinal system has almost disappeared, while most of the radioactivity stays in the inflamed tissues and kidneys. The high kidney activity can be due to an entero-hepatic cycle. Excess of radiolabeled HIgG is trapped

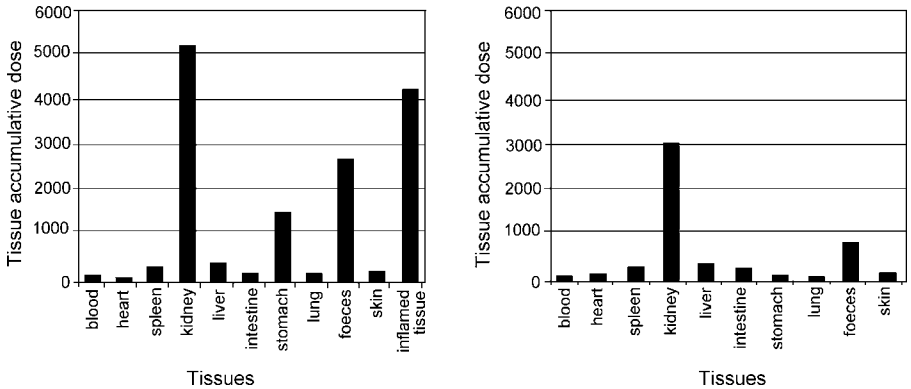

Fig. 6. Injected dose per gram of organs of normal (right) and inflamed (left) rats 24 h after IV injection of high dose ^{111}In-DTPA-IgG (4.4 MBq, 0.1 ml) (n = 3).

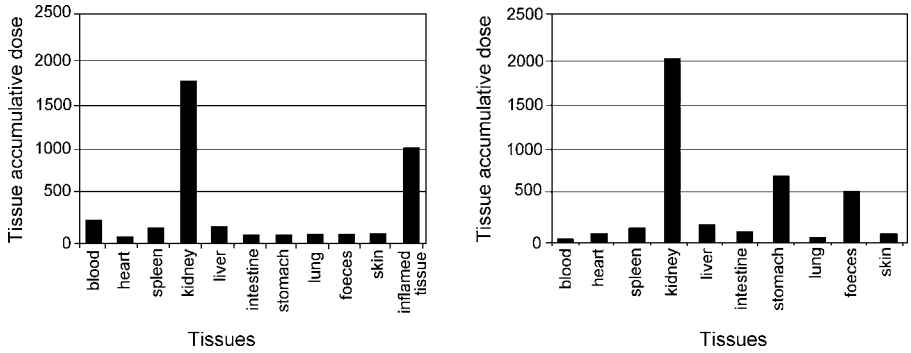

Fig. 7. Injected dose per gram of organs of normal (right) and inflamed (left) rats 110 h after IV injection of high dose ^{111}In-DTPA-IgG (4.4 MBq, 0.1 ml) (n = 3).

in liver after couple of hours like any other protein in the mammals and finally is broken down. The free indium is then excreted *via* hepatobiliary tract into GI system. In the next step, ^{111}In is re-absorbed through intestine and circulate in blood stream. Finally, it is excreted through urinary tract even after 110 h like any other cation.

From these data, it is understandable that 110 h is a rather suitable time for SPECT studies *in vivo* due to high target accumulation. On the other hand, at this dosage possible abdominal inflammations cannot be diagnosed due to the high gastrointestinal activity. The conclusion of the latter must be checked by further experiments on other animal models.

Total labeling and formulation of ^{111}In-HIgG took about 60 min, with a yield of 99%. A suitable specific activity product was formed *via* insertion of ^{111}In cation. No unlabeled and/or labeled conjugates were observed upon RTLC analysis of the final preparations. The radiolabeled complex was stable in mice serum for at least 24 h and no significant amount of free ^{111}In was observed. Trace amounts of ^{111}In-indium chloride ($\approx$ 1%) were detected by TLC. The final preparation was administered to normal and turpentine oil-treated rats and biodistribution of the radiopharmaceutical was checked 1 and 4 days later. In contrast to other labeled immunoglobulins, ^{111}In-DTPA-HIgG, is a suitable inflammation detecting radiotracer with a rather long half-life.

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