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ORIGINAL PAPER

Preparation and biodistribution of ^{99m}Tc-IgG-HYNIC in normal rats

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Abstract. Human gamma globulin can be labeled by a direct or indirect method of radiotracer incorporated in a protein molecule. In this indirect method hydrazinonicotinic acid (HYNIC) is used which saves the structure and biological activity of the protein. Our goal was the efficient labeling of the human gamma globulin and evaluation of its biodistribution in different organs which can be used on experimentally induced infection causing inflammation. Immune globulin is mixed with s-hynic and IgG-hynic is developed using sidle A-lyzer and stored at –20°C which can be used at least for six months and then Sn-tricine kit is prepared which is used for ^{99m}Tc labeling. Efficiency of ^{99m}Tc-IgG-hynic labeling at pH 6.4 was very much dependent on ligand (hynic) and coligand (tricine) presence in the reaction mixture. Radiochemical purity was more than 90% in the kits prepared. Serum stability study showed no decomposition of ^{99m}Tc from the complex. The biodistribution studies showed the highest percentage ID/organ in the blood, liver and kidney, respectively. A human gamma globulin was successfully labeled through hynic to ^{99m}Tc by an indirect method with high radiochemical purity.

Key words: ^{99m}Tc • HIG • HYNIC • IgG-hynic • infection • inflammation

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Introduction

Over the last decades several radiopharmaceuticals have been developed for the detection of infection and inflammation and some have found their way into clinical practice and are routinely used for evaluation of infectious and inflammatory diseases [7, 28, 38, 41]. Radiopharmaceuticals currently used for infection and inflammation are, ^{67}galium citrate [23, 39, 50], radiolabeled leucocytes [17, 31, 40], labeled anti-granulocyte antibodies [8, 14, 49], radiolabeled non-specific human immunoglobulin G(HIG) [15, 26, 37, 45, 47, 50], chemotactic peptides [2–5, 18, 19, 21, 55], interleukines [13, 43, 48, 51–54], radiolabeled liposomes [16, 46] and Platelet factor 4 [34]. Initially, it was believed that human immunoglobulin was retained in inflammatory foci due to interaction with Fc gamma receptors [22], but studies have shown that radiolabeled HIG is retained in infectious foci by non-specific extravasation because of locally enhanced vascular permeability [20]. HIG has been tested for localization of musculoskeletal infection [35], in pulmonary infection [36], and abdominal inflammation [32]. In a comparative study it was shown that labeling HIG through hydrazinonicotinic acid (hynic) by using ^{99m}Tc has a similar characteristic to that of ^{111}In-HIG which in most cases can replace ^{111}In-labeled compound [15]. The radioisotope ^{99m}Tc is the radionuclide of choice in diagnostic nuclear medicine. Current design

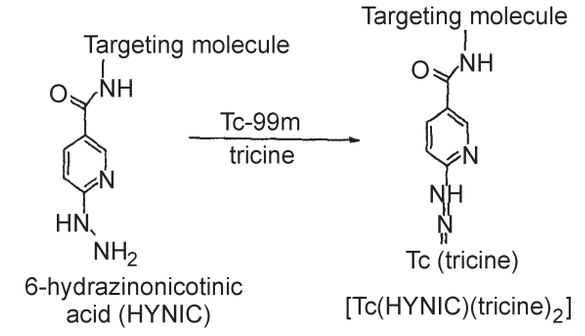

Fig. 1. Conversion of 6 hydrazinonicotinic acid in presence of ^{99m}Tc and tricine.

of new ^{99m}Tc diagnostic agents has concentrated on the development of target specific via the bifunctional approach, this design approach involves the ^{99m}Tc labeling of target or receptor specific molecules via bifunctional chelator which enables a rapid and efficient labeling of targeting molecules such as proteins [10] antibodies [26] peptides [9, 18] or other biomolecules with ^{99m}Tc via hynic. The 6-hydrazinonicotinic acid (Fig. 1) is a bifunctional molecule capable of bonding to lysine residues of peptides or proteins at one end and to ^{99m}Tc on the other, but modification of peptides or proteins is involved with succinimidyl 6-hydrazinonicotinate hydrochloride. The resulting modified molecule can be rapidly labeled with ^{99m}technetium with a high radiolabeling yield.

Experimental

Human immunoglobulin G was obtaind from Baxter (Austria), slide A-lyzer dialysis cassette Pierce #66453 (USA), succinimidyl hydrazinonicotinic acid (s-hynic) from Sololink (USA), Millipore filter (USA), ITLC silica gel strips from Pal Gelman (USA) and the rest of the chemicals used were either from Merck (Germany) or Fluka (Switzerland).

IgG conjugation

The conjugation was done according to the method described by Abrams [1], briefly HIG is dissolved in a final concentration of 50 mg/ml, followed by extensive dialysis (sidle A-lyzer cassette, molecular weight cut off, 10 KD, this device is used for dialysing samples). Low molecular weight contaminant removal, buffer exchange, desalting and concentration can be accomplished and also maintaining the highest sample retention with this device for 24 h in a sterile container against 0.9% NaCl at 4°C under constant stirring, refreshing it 4 times and then 3 ml of the content with final concentration of 30 mg/ml is adjusted to 5 ml and then 1/10 vol. of 1.0 M $NaHCO_3$ is added to IgG and sterilized by membrane filtration. 4 molar excess of freshly dissolved s-hynic was solubilized in 100 µl of dry DMSO and added dropwise to stirred IgG in 10 portions (portion/min) after incubation in the dark for 30 min. 3 ml of the solution is taken and the pH is adjusted to 6.4 using 0.15 M Na/acetate and injected into another sidle A-lyzer and kept over night against Na/acetate buffer pH 6.4 at 4°C as described above. After drawing up the content, it is passed through a 0.22 µm filter and pH is adjusted to 6.4 using the same buffer and the final concentration is made to 4 mg/ml. The solution is divided into 0.5 ml aliqoutes and stored frozen at –20°C until used. Tricine-$SnSO_4$ kits were prepared containing 100 mg tricine which is dissolved in sterile distilled water and oxygen is removed by applying N_2/argon through it. Then 10 mg of $SnSO_4$ in 0.5 ml of 2.0 M HCl is added to the tricine solution applying N_2/argon again through it, the pH is then adjusted to 4.5 as this is the best pH to avoid turbidity and the total volume is made to 10 ml with 0.9% NaCl which is passed through 0.22 µm filter and divided into 10 vials and stored frozen at –20°C until used.

Methods of ^{99m}Tc-IgG-hynic preparation

An IgG-hynic vial is thawed and Sn-tricine kit is dissolved in 5 mL of sterile 0.9% saline solution. 50–60 µl of the freshly dissolved Sn-tricine is added to the IgG-hynic vial and then 1110 MBq of freshly eluted ^{99m}Tc eluate is added and incubated for 15 min at room temperature in the dark.

Radiochemical purity of ^{99m}Tc-IgG-hynic

After the incubation period 2–4 µl of the radio complex (^{99m}Tc-IgG-hynic) is spotted on the ITLC-SG strips. The strips were run using 0.15 M citrate buffer with pH 5.0 as the mobile phase. Radiochromatography was performed by a Bioscan instrument AR-2000 (Paris, France). The R_f values for the labeled compound is about 0.1–0.2 whereas the free ^{99m}Tc moves to the top (R_f = 0.9–1.0).

Colloid determination

Colloid could be technetium oxide or technetium hydroxide, to check these ITLC-silica gel strips were impregnated with 1% human serum albumin (HSA) solution. The strips were soaked in HSA solution for 10 s, stored at 40°C in an oven or air dried and were stored in an air tight container or refrigerator and used within a week. Albumin saturated strips should be used along with sample strips (^{99m}Tc-IgG--hynic). The solvent used for colloid determination is ammonia:ethanol:water in a ratio of 1:2:5 and freshly prepared each time. The labeled compound moves to the top (R_f = 0.9–1.0), whereas the colloid remains at about 0.1–0.2.

Serum stability studies

7 ml of blood is centrifuged at 3500 rpm for 5 min and then 1 ml of serum is withdrawn. 100 µl of the Tc^{99m}-IgG-hynic was added to 1 mL of serum/PBS and incubated at 37°C up to 4 h. Aliqouts of the reaction mixture were diluted 1:10 with PBS (pH 7.4) and analyzed by ITLC with citrate buffer at 1, 2 and 4 h.

Hynic conjugation

4-nitrobenzaldehyde is an aromatic aldehyde reagent, which is used to quantify the molar substitution ratio of hynic modified protein. The number of hydrazide groups introduced was measured from the absorbance change at 340 nm after reaction of the modified protein with 0.5 mM p-nitrobenzaldehyde in pH 4.73 acetate buffer containing 2.5% acetonitrile for 5 h at 25°C to form hydrazone [25].

Animal model

Normal wistar rats of any sex weighing 180–220 g were used and after anaesthetizing the animals (10 at each time point) by ether in a closed jar they were injected the prepared complex (100 μCi/100 μl) through caudal tail vein and sacrificed at 4 and 24 h intervals. The organs removed were the liver, blood, kidney, spleen, stomach, lung, muscle and thyroid gland, washed in normal saline, dried on a filter paper, weighed and counted. The activity is counted in a well type counter (Canberra, USA) at 140 KeV. The data are expressed as percent injected dose per organ (%ID/organ) [6].

Results

In our study radiochemical purity, determined as the mobile phase at pH 6.4, was at least 95% (Fig. 2) and the colloid less than 5% using an ethanol : ammonia : water mixture indicating that the amount of radiolabeled colloid is minima (Fig. 3). Free technetium moves to the top using citrate buffer (Fig. 4). Serum stability was recorded up to 4 h (1, 2 and 4), and the results for ^{99m}Tc-IgG-hynic were showing stability at the end of all the tests studied (Fig. 5). To determine the number of conjugated hynic per hynic-conjugated proteins p-nitrobenzaldehyde is used, as it reacts with hynic-IgG quantitatively with generation of a specific hydrazone. There was no decomposition of ^{99m} Tc from the complex during the course of the studies and the radiochemical purity of the complex remained not less than 99% for 4 h under physiological conditions. The biodistribution study as Fig. 6 shows is as follows, the highest

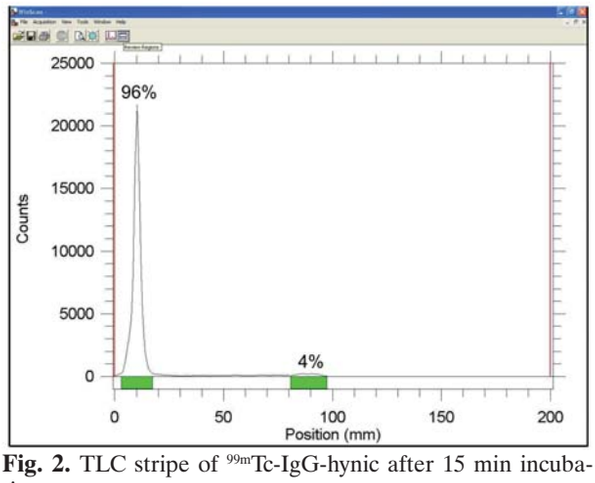

Fig. 2. TLC stripe of ^{99m}Tc-IgG-hynic after 15 min incubation.

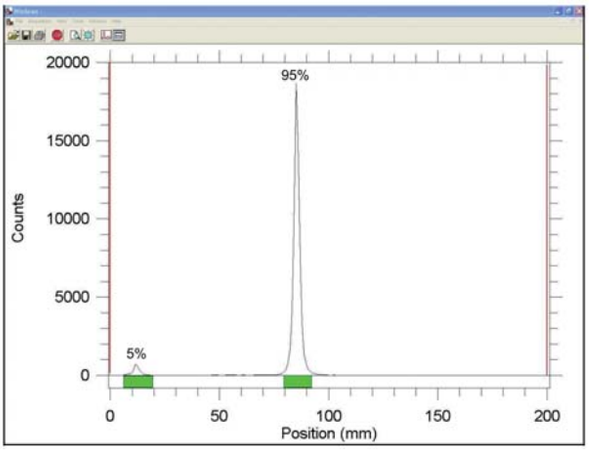

Fig. 3. TLC stripe of colloid content of the preparation.

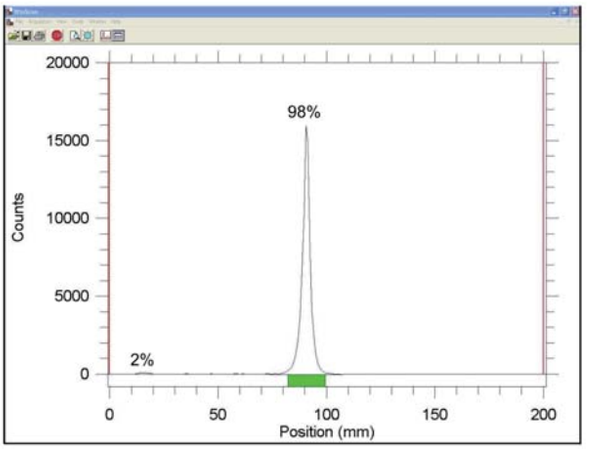

Fig. 4. TLC stripes of ^{99m}Tc after 15 min incubation using citrate buffer.

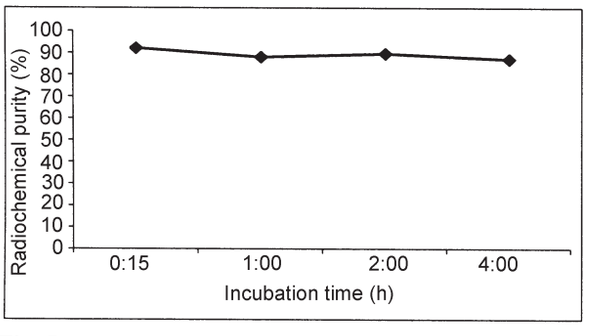

Fig. 5. Serum stability test at 0.15, 1, 2 and 4 h.

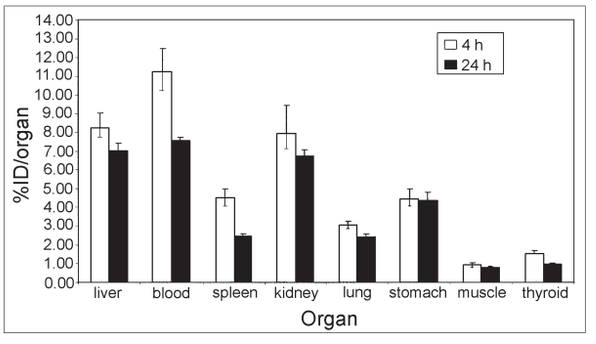

Fig. 6. Biodistribution of Tc^{99m}-IgG-hynic in different organs taken at 4 h and 24 h post injection.

%ID/organ were observed in the blood, liver, kidney, spleen, stomach, lungs, thyroid and muscle respectively at 4 and 24 h intervals.

Discussion

In the development of a new ^{99m}Tc radiopharmaceutical several factors such as biological efficacy and a kit formulation is required to satisfy the clinical requirements. The bifunctional chelating approach appears to be the most suitable for the preparation of the ^{99m}Tc-labeled peptide or protein radiopharmaceuticals especially those for the clinical use, because of their convenience, well defined labeling chemistry and feasibility of kit formulation [29]. A kit contains the substance to be labeled, a ligand or a bifunctional chelator and a reducing agent such as stannous sulfate. The new radiopharmaceutical should have high a radiochemical purity not less than 90% and a high solution stability with a shelf life preferably not less than 6 h. The hynic group is of particular interest as it can be easily labeled with very high efficiency, rapid as well as high yield radiolabeling since the hynic can only occupy one or two coordination sites to a coligand such as tricine. Larsen [26] has shown ^{99m}Tc-tricine is a useful precursor complex for the radiolabeling of hynic-protein conjugates. Hynic have been used by different workers in the labeling of a variety of targeting molecules from polycolonal IgG [27, 47], chemotactic peptides [2, 18, 21, 55], somatostatin analogues [11], and receptor antagonists [12]. In our study tricine is used as a coligand. In the formed ^{99m}Tc-hynic-IgG, the technetium form is coordinated by two tricine molecules and the terminal N atom of the hydrazine group of hynic [30], moreover the influence of the conjugation ratio on the number of introduced hynic groups per protein molecule has been investigated for polycolonal IgG [47], a fourfold molar excess of s-hynic is required to incorporate one hynic group per IgG molecule at a protein concentration of 4.6 mg/ml [42]. In our study the protein concentration is fixed at 4.0 mg/ml and the hynic conjugation analysis using hydrazone has shown that at least one hynic group is incorporated into IgG molecule.

As a comparison with different workers, Abrams *et al*. [1] observed greater ^{99m}Tc-IgG accumulation in lung, liver and spleen than ^{111}In-IgG in disinfected rats. Mishra *et al*. [33] showed the highest %ID/organ in mice at 4 h in the liver, blood, kidneys and spleen, respectively and the values were as follows: 10.2, 9.7, 5.39 and 2.3. Observations of Dams *et al*. [15] in granulocytopenic rats showed %ID/g in the spleen, blood, lung, liver, and kidney as follows: 7.3, 5.9, 5.6, 4.1 and 3.2.

Rennen *et al*. [44] have stated that both ^{111}In and ^{99m}Tc labeled HIG have shown blood clearance and physiological uptake in the liver, spleen and kidneys. Karczmarczyk *et al*. [24] showed the highest %ID/g in mice at 4 h in the blood, liver, kidney and lungs and the values were: 9.0, 3.0, 2.5 and 1.75. Considering the above observations by different workers and comparing their results, although there are some differences, some results also show similarities.

Conclusion

^{99m}Tc-IgG is successfully labeled using succinimidyl hydrazinonicotinic acid as a bifunctional molecule capable of bonding to lysine residues of protein with a high radiolabeling yield. The radiopharmaceutical is based on human IgG-hynic derivative and labeled at room temperature at pH 6.4 for 15 min in the presence of tricine as chelator and $SnSO_4$ for pertechnate reduction. The radiochemical purity obtained for different batches were at least 90% and the colloid content determined were not more than 5%. The biodistribution studies showed the highest %ID/organ in the blood, liver and kidney at 4 and 24 h intervals. Labeling of hIgG via hynic and Sn-tricin is a viable and plausible method which provides a quality radiopharmaceutical with a good *in vitro* and *in vivo* stability. A human gamma globulin derivative IgG-hynic labeled with ^{99m}Tc by an indirect method with a high radiochemical purity can be used as a basic compound of formulation for infection and inflammation scintigraphy in infected rats.

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