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Technetium-99m-Human IgG Radiopharmaceuticals: Preparation, Biodistribution and Infection Imaging in Mice

WIDYASTUTI WIDJAKSANA*[1], ANNA ROSELIANA[1], AGUS ARIYANTO[1], SRI AGUSWARINI[1], MARIALINA[2], GINA MONDRIDA[1]

[1]Center for Radioisotopes and Radiopharmaceuticals, BATAN, Kawasan Puspiptek, Serpong, Banten 15310.
[2]Center for Application of Isotope and Radiation Technology, BATAN, Jl Raya Lebak Bulus, Jakarta Selatan.

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Abstract: Technetium-99m-Immunoglobulin-G preparation and analysis were carried out using human immunoglobulin-G (IgG) which was conjugated with hydrazinonicotinamide (HYNIC) prior to labeling with technetium-99m (^{99m}Tc), and the HYNIC-IgG molecules were stabilized with a co-ligand, tricine. Tricine was prepared both in the form of lyophilized kits and in frozen solutions and their stabilities were compared. The effect of pH on the labeling efficiency was also studied. Characterization of native IgG as well as the radiolabeled IgG were carried out using size exclusion HPLC, whereas the labeling efficiency of ^{99m}Tc-HYNIC-IgG was determined using thin layer and paper chromatographic methods. The stability of radiolabeled ^{99mTc}-HYNIC-IgG at room temperature as well as in human serum were investigated by observing the radiochemical purity within 4 hours *in vitro*. The shelf-life of unlabeled HYNIC-IgG stored at −40°C and tricine kits stored at 4°C were determined. Biodistribution of ^{99m}Tc-HYNIC-IgG in healthy mice and in infection-induced mice and rats were also studied. The HPLC results showed that the native and radiolabeled IgG had similar retention times, which indicated that conjugation and radiolabeling processes did not affect the integrity of the IgG molecules. The radiochemical purity of 99mTc-HYNIC-IgG was high — more than 90% — without purification step, and the preparation was stable up to 4 hours. Tricine kits prepared at pH 3 was proven to produce clear solution and high labeling yield, while pH 4 produced slight opalescence solution which turned to turbid after a few hours. Biodistribution studies in healthy mice showed an obvious uptake in liver but normal distribution in other tissues, while biodistribution in infection-induced mice showed significantly different uptake between infected tissues, i.e higher than normal tissues. Blood clearance was achieved within 2 hours and excretion via urine and faeces were observed within 24 hours. It is concluded that the preparation using human IgG showed high uptake in the infection site, and the ^{99m}Tc-HYNIC-IgG can be a promising radiopharmaceutical for infection or inflammation imaging.

Key words: Antibody, immunoglobulin-G , Technetium-99m, HYNIC, infection, inflammation.

INTRODUCTION

INFECTIOUS diseases caused by bacteria, fungi and virus are common in tropical countries including Indonesia, which should be effectively treated, or otherwise, will cause resistency to some antibiotics.

Prior to treatment with an antibiotic, the extent of infection should be confirmed by an accurate diagnostic method. Although the microbiological examination is considered as a gold standard method, the method cannot be used to inform the location of infection in the body. Some other modalities for detecting infection are X-ray, CT scan, USG, and MRI. Nuclear techniques for the detection of infection using radiopharmaceuticals have been developed for decades, although many of them cannot distinguish between infection and inflammation nor specifically determine the type of microorganisms. Some radiopharmaceuticals already used for infection imaging are ^{67}Ga-citrate, ^{111}In-leucocytes, ^{99m}Tc- HMPAO-leucocytes, ^{111}In-DTPA-IgG and ^{99m}Tc- IgG. One advantage of using radiopharmaceutical such as ^{67}Ga-citrate, ^{111}In-DTPA-IgG and ^{99m}Tc-HYNIC-IgG is its capability to

* Penulis korespondensi, Hp. 08128405431
e-mail: widi_as2002@yahoo.com

detect abnormality caused by active infection and to distinguish infection or inflammation from tumour, e.g. Kaposi's sarcoma[1].

Human Immunoglobulin-G (IgG) is a natural defense protein against infection which is normally found in the human body, called antibody.

The molecular weight of IgG is around 150,000 Da, and the chemical structure of this protein is unique that consists of 2 chains of protein: light chain and heavy chain, linked by a disulphide bond. Each chain is divided into 2 domains: variable domain that has various amino acids sequence, and constant domain that has relatively constant amino acids sequence. Variable chains reflect in different specificity of the antibody against microorganism, whereas constant chains act as a mediator for a secondary function of immunology system. The variable domain has primary amine terminal while costant domain has carboxylate terminal. Human IgG is commonly used in medicines especially to increase immunity in patients. One of IgG brand name in local market is Gamimune N 10% SD (Dipa PharmaLab)[2, 3].

IgG based radiopharmaceuticals labeled with indium-111 (^{111}In) had been successfully developed previously as infection/inflammation imaging agents. However, since this radionuclide is produced in a cyclotron machine, and an imported ^{111}In is very expensive, it is not feasible to develop ^{111}In-IgG in Indonesia[1,5]. Another radionuclide which is available in Indonesia and is commonly used with comparable performance is technetium-99m (^{99m}Tc).

^{99m}Tc-HYNIC-Immunoglobulin-G radiopharmaceutical could be developed in Indonesia since the antibody has been widely used in patients with no adverse reactions, the price is moderate, and ^{99m}Tc radionuclide is regularly used and locally supplied (^{99}Mo/^{99m}Tc generator, Batan Technology Inc.).

Immunoglobulin-G (IgG) can be directly labeled with ^{99m}Tc or by using a linker called 'bifunctional chelating agent'. In direct labeling method a mild reducing agent is required to break the disulphide bond, resulting in releasing free thiols onto which ^{99m}Tc will bind; whereas in indirect labeling method a ligand is required to act as a linker between the radionuclide and IgG molecule. The ligand should has a carboxylic group to bind with a primary amine terminal of the IgG, and should also has a Lewis acid group which bind the ^{99m}Tc to produce a stable chelate complex. Bifunctional chelators (BFC) which are commonly used for this purpose are MAG3, DTPA, DOTA and HYNIC[6].

N-hydroxysuccinimide hydrazinonicotinamide (NHS HYNIC) has been widely used in the development of peptide based radiopharmaceuticals such as HYNIC-tyrosine octreotide, which is used as a neuroendocrine tumor imaging agent. HYNIC does not provide all of the coordination sites necessary for full complexation of the technetium, therefore it must be used in conjuction with a 'co-ligand' which will complete the co-ordination sphere, such as tricine, EDDA and nicotinic acid[4, 6].

In this experiment the NHS HYNIC was chosen as the bifunctional chelator, since it has provided efficient and stable labeling of various proteins, and has also been widely used in radiopharmaceutical developments.

To obtain good quality radiopharmaceuticals, the labeling methods should be optimized, and all requirements for an infection or inflammation imaging agent should be complied, including its effectivity in experimental animals.

Tricine was used as the co-ligand and tin (II) sulphate as the reducing agent for $^{99m}TcO_4^-$ (VII) to a lower oxidation state (IV). Tricine and tin sulphate were prepared in a 'ready to use' kit, usually lyophilized. In the process, however, the solution is easily become turbid when the pH is increased to 4 as a recommended pH for labeling reaction. Therefore, it is necessary to find an optimum condition for producing a clear solution of Sn(II)-tricine preparation, and later to produce a high labeling yield of ^{99m}Tc-HYNIC-IgG.

MATERIALS AND METHODS

MATERIALS. Material used in the experiment were Human IgG (Gamimune-N 10% SD, Dipa Pharmalab), N-hydroxy-succinimide hydrazino nicotinamide (NHS-HYNIC, Soluilink), tricine (Aldrich), tin (II) sulphate (Aldrich), sodium ^{99m}Tc pertechnetate (Batan Technology), dialysis cassettes (cut-off 10,000 Da, Pierce), dimethylsulphoxide (DMSO,Aldrich), ITLC-SG (PALL), *Staphylococcus aureus* and *Escherichia coli* cultures of 10^9 cfu (PATIR, BATAN), mice, Wistar rats, rabbits (BBPMSOH), TLC scanner (Veenstra Instrument), freeze dryer (Labconco) and animal gamma camera.

METHODS. Preparation of ^{99m}Tc-HYNIC-IgG comprises of several steps, starting from conjugation of HYNIC to IgG, preparation of Sn-tricine kits, labeling of HYNIC-IgG with ^{99m}Tc, analysis of ^{99m}Tc-HYNIC-IgG, animal study including biodistribution study in healthy and in infection-induced mice, and excretion study.

Conjugation of IgG with HYNIC. IgG was dialyzed in saline overnight at 4°C (the medium was refreshed 4 times) prior to conjugation. Conjugation

of HYNIC-IgG was carried out by mixing 150 mg of IgG with 3 molar folds of NHS-HYNIC (1 mg HYNIC in 0.1 ml DMSO) at room temperature in the dark for 30 minutes, diluted with 0.15 M acetate buffer of pH 6.4, then followed by purification using dialysis cassette in acetate buffer at 4°C for 2 days during which the medium was refreshed 4 times. Purified HYNIC-IgG was diluted with acetate buffer to get concentration of 4 mg/ml, and dispensed into vials 0.5 ml each for a single labeling.

Preparation of Sn-Tricine kits. 100 mg of tricine was dissolved in water, added with tin (II) sulphate (10 mg in 1 ml of 1 N HCl) under nitrogen atmosphere, pH was adjusted to 2.5; 3.0; or 4.0, then 1 ml of each were dispensed into 10 ml vials, and were lyophilized in freeze dryer for 24 hours, and stored at 4°C in a refrigerator. If the kits will not be lyophilized then they should be stored at –20°C or lower. Each vial contains 10 mg of tricine and 1 mg of $SnSO_4$. The Sn-Tricine kits then were used in the labeling of HYNIC-IgG with ^{99m}Tc as described below.

Labeling of HYNIC-IgG with ^{99m}Tc. Initiallly, the Sn-tricine kit was reconstituted with 5 ml of saline solution, then added to a vial containing 2 mg of HYNIC-IgG followed by an addition of 20 mCi of ^{99m}Tc pertechnetate. The mixture was allowed to react at room temperature for 15 minutes.

Labeling yield of HYNIC-IgG with ^{99m}Tc. Radiolabeling yield of HYNIC-IgG was determined by paper chromatographic (PC) method and thin layer chromatographic (TLC) method. Whatman 1 chromatographic paper was used as the solid phase and acetone as the mobile phase, to determine % free ^{99m}Tc-pertechnetate ion as an impurity that will be eluted with the solvent front. Other possible impurity, ^{99m}Tc colloid, was determined by thin layer chromatographic method (TLC) with an ITLC-SG impregnated with 1% human serum albumin as the solid phase and a mixture of ethanol-ammonia-water (2:1:5) as the mobile phase, in which the ^{99m}Tc colloid impurity will remain at the origin. Another possible impurity, ^{99m}Tc tricine (which is usually called ^{99m}Tc co-ligand) was determined using ITLC-SG and 0.15 M citrate buffer pH 5 as the eluant, in which the ^{99m}Tc tricine impurity, together with the free ^{99m}Tc-pertechnetate, will migrate with the solvent to a distance with Rf of 0.8. Then, the labeling yield is calculated = [100% - total amount of impurities (% ^{99m}Tc colloid + % free ^{99m}Tc-pertechnetate + %^{99m}Tc tricine)]. There was no purification step to be carried out at this stage.

Stability test. The stability of both unlabeled HYNIC-IgG and radiolabeled ^{99m}Tc-HYNIC-IgG were investigated during storage for certain periode of time. The HYNIC-IgG solution was stored at –40°C, frozen Sn-tricine kits were stored at –20°C, and lyophilized Sn-tricine kits were stored at 4°C. Every week each of them were sampled and analyzed for the radiochemical purity after labeling with ^{99m}Tc using provided protocols. Stability of the radiolabeled HYNIC-IgG was investigated by incubating 2 mCi of ^{99m}Tc-HYNIC-IgG in 1 ml of fresh human serum at 37°C, and samples were taken every hour for the radiochemical purity test. Similar investigation was carried out in phosphate buffer saline (PBS) of pH 7 as control. Stability of the radiolabeled HYNIC-IgG at room temperature was also investigated. The radiochemical purity of a preparation for parenteral use should be higher than 90%, and the impurity should not exceed 5%.

Biodistribution study. Biodistribution study of the ^{99m}Tc-HYNIC-IgG was carried out in infection-induced mice and also in healthy mice as control. Prior to biodistribution study each mouse was injected intramuscularly with 0.2 ml of *Escherichia coli* suspension (10^9 cfu per ml) on the right leg, and allowed to swell for 24 hours. Two hundred μCi in 200 μl of ^{99m}Tc-HYNIC-IgG was injected through each mouse tail vein. After 1 hour and 24 hours the mice were sacrificed, and the organs including blood, muscles and bone were taken, weighed and counted for radioactivity. For gamma camera imaging a bigger animal was required to get obvious image so the investigation were carried out in rats which did not need to be dissected. Blood clearance was observed by taking out the blood at 5, 10, 30, 90 and 120 min. and measured the radioactivity. Excretion through kidneys and gastrointestines were carried out in rabbits using standard method by collecting the urine/feces in metabolic cages within 3 days.

RESULTS AND DISCUSSION

Conjugation of HYNIC with IgG. Human Immunoglobulin-G (IgG) was used in the investigation with consideration that the substance is naturally found in human body and over expressed in infection and inflammation sites. The substance is available in local market, one of which is Gamimune $N^{R(2)}$. IgG was labeled indirectly using a bifunctional chelator, N-hydroxy succinimide-hydrazinonicotinamide(NHS-HYNIC), which acts as a linker to form a predictable IgG-complex without altering the antibody molecule. Conjugation was successfully done with high yield and the conjugates was characterised using size exclusion HPLC at 280 nm (SE-HPLC) and showed that both IgG and

HYNIC-IgG produced a single peak at minute-6, indicating that both have similar molecular weight (Figure 1). Therefore it could be accepted that the conjugation process did not alter the integrity of the IgG molecule.

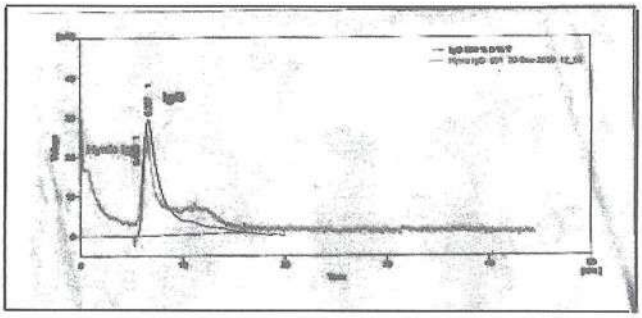

Figure 1. Chromatogram of IgG and HYNIC-IgG from size exclusion HPLC.

Preparation of Sn-Tricine kits. pH adjustment in the preparation of Sn-tricine kits should be carried out carefully or otherwise turbidity will occur. In other words, it is important to understand the relationship between pH and the labeling efficiency. In the preparation of Sn-tricine kit, the pH was adjusted to 4 as recommended in the protocol, however, turbidity was occurred. Further investigation showed that pH adjustment to 2.5 or 3 produced clear solutions, and also produced high radiochemical purity of ^{99m}Tc-HYNIC-IgG (Table 1, Figure 2). The turbidity was likely occured when Sn-tricine solution was at pH 4 and in contact with air after some time due to the formation of unsoluble complex of Sn(OH)tricine. Therefore, Sn-tricine kits were later prepared at pH 3 as selected method, dry (lyophilized form) and should be used immediately after reconstitution.

Table 1. Labeling yield and appearance of ^{99m}Tc-HYNIC-IgG using Sn-tricine kits of various pH (n=3).

Initial pH	pH after labeling	Appearance	% labeling yield
2.5	6	Clear	93.12 ± 1.6
3	6	Clear	94.67 ± 1.2
4	7	Opalescent	95.17 ± 1.18

Labeling of HYNIC-IgG with ^{99m}Tc. The radiochemical purity of ^{99m}Tc-HYNIC-IgG prepared using Sn-tricine of pH 3 indicated that the preparation method has good reproducibility with SDR of 1.30

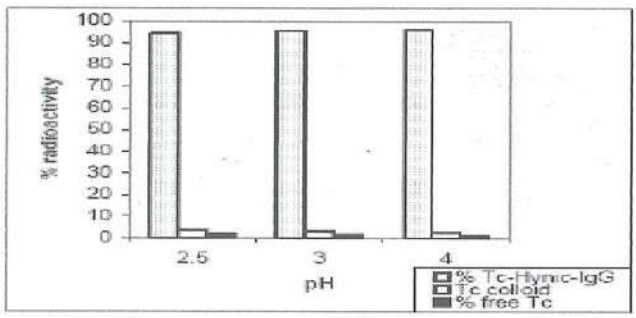

Figure 2. Radiochemical purity of ^{99m}Tc-HYNIC-IgG using Sn-tricine kit in various pH.

(Table 2). Average radiochemical purity of 94.73% was good considering that the preparation was carried out without purification step, as well as the short half-life nature of ^{99m}Tc.

Table 2. Labeling yield of 99mTc-HYNIC-IgG using Sn-tricine kits prepared at pH 3.

Components	n	Average	SD
Free ^{99m}Tc + ^{99m}Tc tricine (%)	7	2.23	1.01
^{99m}Tc colloid (%)	7	3.04	1.41
^{99m}Tc-HYNIC-IgG (%)	7	94.73	1.47

Stability test. The stabilities of IgG and HYNIC-IgG in phosphate buffer saline solution at room temperature, and in fresh human serum at 37°C as simulation of body environment were observed for 5 hours. The radiochemical purity of ^{99m}Tc-HYNIC-IgG at room temperature was slightly decreased after 3 hours, with a concomitant increase of free ^{99m}Tc pertechnetate ion and ^{99m}Tc colloid fragments (Table 3). ^{99m}Tc-HYNIC-IgG incubated in fresh human serum at 37°C for 5 hours was more stable than in PBS solution (Figure 3).

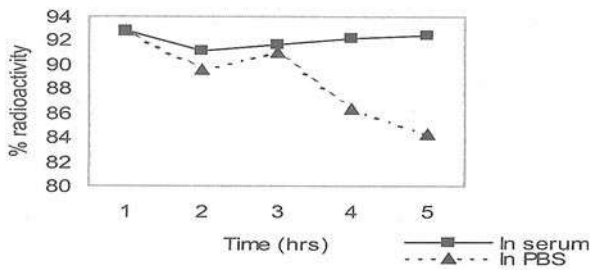

Figure 3. Stability of 99mTc-HYNIC-IgG in serum and in PBS (n=2).

Sn-tricine kits both in solution or lyophilized form were stable over 8 months after preparation, as indicated by its ability to promote the formation of ^{99m}Tc-HYNIC-IgG. The HYNIC-IgG stored at –40°C was still stable over 13 months after preparation. This observation will be continued until its ability to produce radiochemical purity of ^{99m}Tc-HYNIC-IgG decreased to less than 90% (Figure 4).

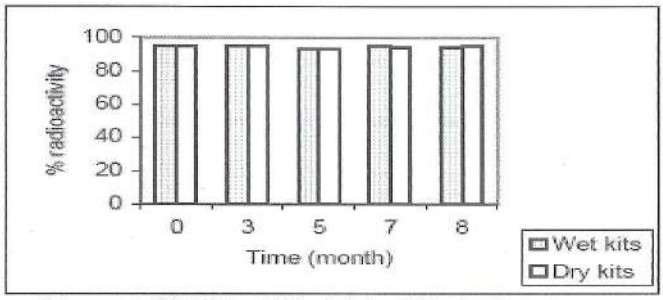

Figure 4. Stability of Sn-tricine kits during storage.
Note : HYNIC-IgG used were from the same batch

Biodistribution study. ^{99m}Tc-HYNIC-IgG was administered to experimental animals to investigate the pharmacokinetic profile and the uptake in infection or inflammation site. Mice were used for biodistribution study, Wistar rats were used for gamma camera imaging and rabbits were used for blood clearance and excretion study. Biodistribution of ^{99m}Tc-HYNIC-IgG 1 hour post injection in healthy mice showed accumulation of ^{99m}Tc-HYNIC-IgG in the blood, kidneys, stomach, liver, and muscles. Accumulation in the stomach indicated an existence of free ^{99m}Tc-pertechnetate

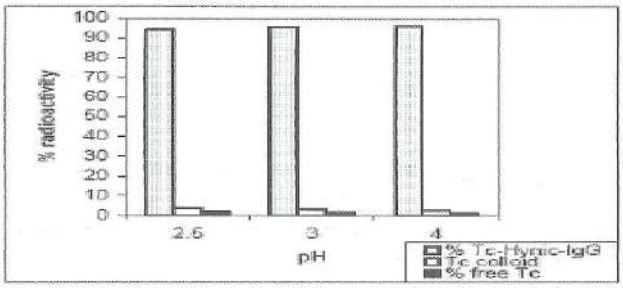

Figure 5. Biodistribution of ^{99m}Tc-HYNIC-IgG in healthy mice as control, 1 hour post injection.

ion. The presence of radioactivities in muscles indicated normal circulation of blood, radioactivities in liver and kidneys indicating some degree of initial ^{99m}Tc-HYNIC-IgG excretion (Figure 5), while biodistribution study of ^{99m}Tc-HYNIC-IgG 1 hour post injection in mice infected with *S. aureus* and *E coli* showed higher radioactivity in infected muscles rather than in uninfected ones (Figure 6 and 7).

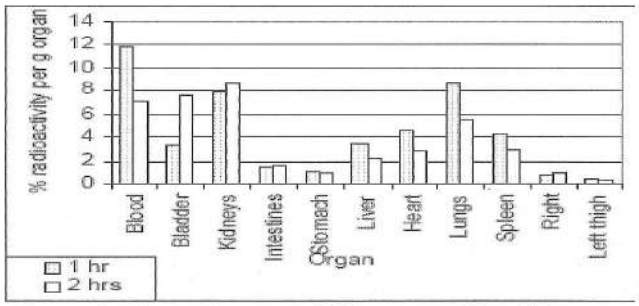

Figure 6. Biodistribution of ^{99m}Tc-HYNIC-IgG in mice infected with *S. aureus* 1 hour post injection.

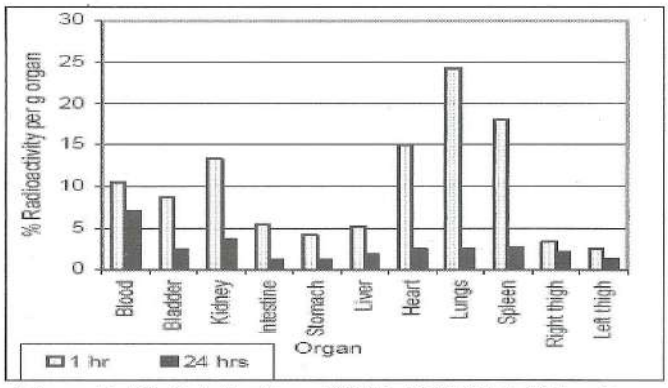

Figure 7. Biodistribution of ^{99m}Tc-HYNIC-IgG in mice infected with *E. coli* 1 hour post injection.

Biodistribution study showed that accumulation of ^{99m}Tc-HYNIC-IgG in the right thigh (infection site) of infected mice was higher than that in the left thigh (uninfected tissue) (Figure 7).

Gamma camera imaging was carried out in rats infected with *Staphylococcus aureus* and *Escherichia coli* on the right thigh. Figure 8a, 9a, and 10a showed the whole body image, whereas Figure 8b, 9b, and 10b showed partial body image of rat thigh, to compare uptakes of the right and left thigh (image above the region of interest was covered with lead shield to increase the image contrast on the region of interest). The images showed that uptake of ^{99m}Tc-HYNIC-IgG in rat infected with *S. aureus* was much higher than that with *E. coli*, which indicating that inflammation caused by *S. aureus* was more severe

than that of *E. coli* (Figure 8, 9, and 10).

Radioactivity in blood was observed 5 minutes post injection and only slightly decreased after 2 hours (Figure 11). Excretion through urinary tract started at 2 hours post injection, and excretion through faeces was first observed at 23 hours post injection. The results of blood clearance and excretion from the body clearly showed that ^{99m}Tc-HYNIC-IgG was washed out from the blood after 2 hours post injection, and remained in some organs up to 24 hours (Figure 11-13).

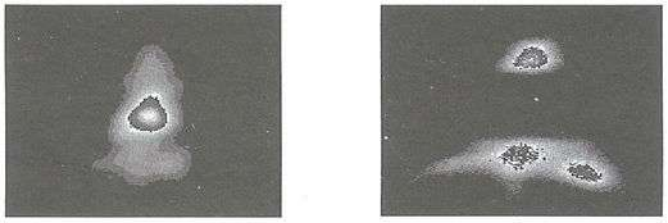

Figure 8 a-b. Gamma camera images of rat infected with *S. aureus*, 1 hour post injection with ^{99m}Tc-HYNIC-IgG.

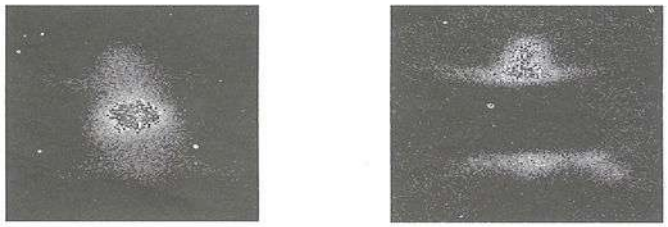

Figure 9 a-b. Gamma camera imaging of rat infected with *E. coli* 1 hour post injection with ^{99m}Tc-HYNIC-IgG.

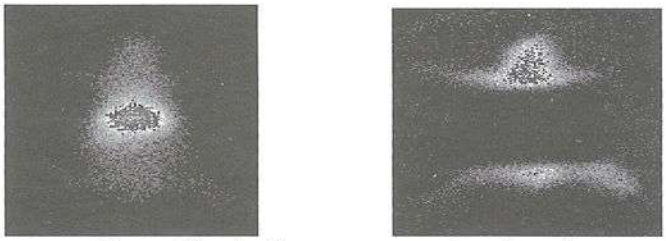

Figure 10 a-b. Gamma camera imaging of rat infected with *E. coli* 24 hours post injection with ^{99m}Tc-HYNIC-IgG.

CONCLUSIONS

Human Immunoglobulin-G can be highly labeled with ^{99m}Tc through a linker NHS-HYNIC and was stable up to 4 hours post labeling. Unlabeled HYNIC-IgG was stable (more than 15 months), as

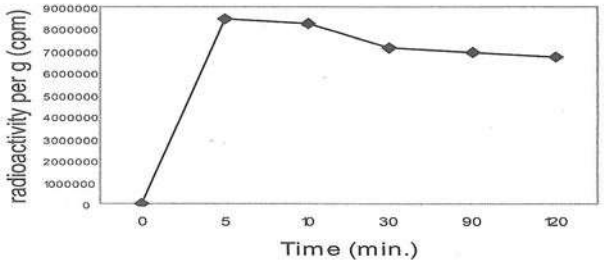

Figure 11. Blood clearance profile of ^{99m}Tc-HYNIC-IgG in healthy rabbits.

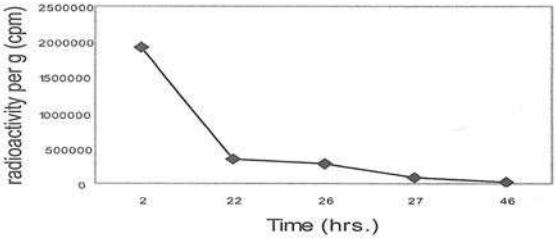

Figure 12. Excretion profile of ^{99m}Tc-HYNIC-IgG through rabbit urine.

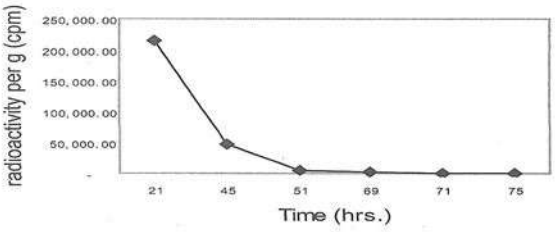

Figure 13. Profile of ^{99m}Tc-HYNIC-IgG excretion through rabbit faeces.

well as Sn-tricine kits (more than 8 months) both in solution form and lyophilized kits. Stability of ^{99m}Tc-HYNIC-IgG in fresh human serum was also stable up to 4 hours, indicating that it will remain intact in the human body at least for 4 hours.

HPLC results showed that the native and radiolabeled IgG have similar retention times, which indicated that conjugation and radiolabeling processes did not affect the integrity of the IgG molecules.

To prevent Sn-tricine kits from becoming turbid, the pH of final Sn-tricine preparation should

be adjusted to between 2,5 to 3, and contact with air should be minimized by thoroughly purging the solution with nitrogen gas, then stored in a lyophilized form.

The effectiveness of ^{99m}Tc-HYNIC-IgG as an infection or inflammation imaging agent had been proven in mice and rats since the preparation showed high uptake in the infection site, and the ^{99m}Tc-HYNIC-IgG can be a promising radiopharmaceutical for infection or inflammation imaging. The next step is clinical studies in patients, after obtaining a permit from the Ethical Commission in hospitals.

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