GOCE DELCEV UNIVERSITY

FACULTY OF MEDICAL SCIENCES

MASTER THESIS / МАГИСТЕРСКИ ТРУД

FORMULATION OF FREEZE-DRIED RADIOPHARMACEUTICAL IMMUNOGLOBULIN G – KIT FOR INFECTION AND INFLAMMATION IMAGING

ФОРМУЛАЦИЈА НА ЛИОФИЛИЗИРАН РАДИОФАРМАЦЕВТИК ИМУНОГЛОБУЛИН Г – КИТ ЗА ВИЗУЕЛИЗАЦИЈА НА ИНФЕКЦИИ И ИНФЛАМАЦИИ

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LIST OF ABBREVIATIONS

BSA	Bovine Serum Albumin
CT	Computed Tomography
FDG	Fluoro Deoxy Glucose
GMP	Good Manufacturing Practice
HIG	Human Immunoglobulin G
HMPAO	Hexa-Methyl Propylene Amino Oxime
HYNIC	Succinimidyl Hydrazi–Nonicotinic Acid
IAEA	International Atomic Energy Agency
IgG	Immunoglobulin G
ITLC-SG	Instant Thin Layer Chromatography – Silica Gel
MDP	Methylene Diphosphonate
MRI	Magnetic Resonance
PBS	Phosphate Buffer Saline
PET	Positron Emission Tomography
RES	Reticloendotheliale System
SPECT	Single Photon Emission Computed Tomography
UV	Ultra Violet
WBC	White Blood Cell

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DEFINITIONS

Conjugation	The chemical attachment of a chelator molecule to an antibody.
Immunoconjugate	The resultant adduct of conjugation.
Labeling	The tagging of a molecule with a radioactive isotope.
Chelation	The complexing of a metal ion with a multi-dentate ligand.
Freeze-dried Cake	The dry product in the vial on completion of freeze-drying.
UV-VIS	Ultraviolet – Visible spectrophotometer.

ABSTRACT

Infectious and inflammatory diseases are the commonest cause of mortality around the world. Rational diagnosis of infectious and inflammatory diseases is important for better disease management outcome.

Nuclear Medicine procedure using radiopharmaceuticals gives both functional and anatomical information. The study objective was to formulate a ready use cold kit of human immunoglobulin IgG to be labeled with 99mTc for the diagnosis of infectious and inflammatory disease. We used both the direct and indirect labeling method.

We obtained better labeling efficiency for the directly labeled kit.

Key words:

Immunoglobulin G, infection, inflammation, kit formulation, Technetium-99m

1. INTRODUCTION

1.1. Background information

Infection and inflammation still remain the major cause of mortality and morbidity in most developing countries despite advances in treatment and diagnosis [1] Appropriate diagnosis of infection and inflammation at an early stage is crucial to the improvement of the efficiency of treatment and prognosis. Different diagnosis modalities such as ultrasound, computed tomography (CT), magnetic resonance (MRI), etc. have been used, which only gives anatomical sites of infection and inflammation foci. Nuclear medicine procedure using radiopharmaceuticals is invaluable in providing both anatomical and functional imaging of infection and inflammation information [2, 3].

Several radiopharmaceuticals have been developed, evaluated and clinically used to identify the site of infection and inflammation in a specific situation. These include ^{99m}Tc-methylene diphosphonate (MDP), ^{67}Ga citrate, radiolabeled leukocytes, FDG positron emission tomography, radiolabeled antimicrobial, radiolabeled antibacterial peptides, radiolabeled monoclonal and polyclonal human immunoglobulin G (antibodies). However, none of them is an ideal radiopharmaceutical for the detection of infection and inflammation [4].

Currently ^{99m}Tc labeled leukocyte radiopharmaceutical is accepted as a gold standard method for the detection of acute infection and inflammation foci. Unfortunately, the method has several disadvantages limiting its application. It requires a complex blood handling procedure that is a risk for contamination of a patient or personnel, additional facility, equipment, as well as trained experts. Furthermore, the accumulation of radioactivity in non-target organs; 99mTc-HMPAO in bowel, and ^{99m}Tc nano-colloid in RES organs gives false positive results. It is contraindicated in patients with neutropenia and infants, as it requires a large volume of blood for the procedure. Another limitation with this method is the condition that affects leukocyte harvesting and function resulting in poor image quality for instance, patient receiving chemotherapy and other immune modulating agent like glucocorticoids [5, 6].

Furthermore, a Radiopharmacy unit in developing African countries is classified under 2a international atomic energy agency Radiopharmacy classification, which restricts to handle white blood cell labeling.

Therefore, there is need for a new agent at least as good as radiolabeled leukocyte, but easier to prepare, and without its inherent risks. Since there is a commercially introduced injectable form of human immunoglobulin (IgG) suitable for intravenous administration for the treatment of immunodeficiency syndrome, it is good to reconsider radiolabeling of IgG for immunodetection of infection and inflammation foci in the situation where the facility, equipment and personnel are not adequate and radiolabeled leukocyte is contraindicated. Radiolabeled IgG was previously used for the detection of infection and inflammation foci, although it is less specific, radiolabeled IgG offers a simple and practical approach for early detection of acute focal infection. In addition, different authors used various techniques for labeling antibodies and obtained promising results.

The present study shows the approach how to formulate a radiopharmaceutical kit for the detection of infection and inflammation foci using human immunoglobulin G polyclonal antibody. The kit labeled with ^{99m}Tc by direct and indirect methods.

2. LITERATURE REVIEW

2.1. Mechanism of Infection and inflammation

Infection is the invasion of an organism's body tissues by pathogenic organisms such as bacteria, virus and fungi and their multiplication, and the reaction of host tissues to these organisms and the toxins they produce [7]. The body may react to infection through inflammation.

Inflammation is the body's response to infectious or noxious stimuli. The main aim of inflammation is to bring defense cells (immune cells) to the area of concern, as well as to inactivate or destroy invaders/pathogens and to begin repair [8]. Sometimes inflammatory reaction may be initiated against normal body without any foreign agents and causes an autoimmune disease such as rheumatoid arthritis.

Inflammation can be acute inflammation or chronic inflammation. When pathogens or any noxious agents infiltrate into the body and contact the tissue, a series of many responses by the body, particularly by the surrounding cells, will occur during inflammation. Many pathogens or inflammation-producing agents contain certain molecules, for instance many pathogenic bacteria express a protein molecule called PAMP, which is recognized by the immune cells in the body such as mast cells and macrophages. When mast cells and macrophages recognize the foreign bodies/pathogens infiltrating into the body, they are triggered to release the inflammatory mediator, histamines and (cytokines, INF-α, & IL-1) respectively, which will cause vasodilation and increased vascular permeability [9].

Vasodilation leads to greater blood flow to the area of inflammation, resulting in the movement of more immune cells to inflamed tissue, redness and heat. Whereas vascular permeability leads to endothelial cells becoming "leaky" from either direct endothelial cells or via chemicals mediators. Another event that occurred during inflammation is exudation in which fluids, proteins, red blood cells, and white blood cells escape from the intravascular space as a result of increased osmotic pressure extravascularly and increase hydrostatic pressure intravascularly.

Cytokines also trigger repair by activating the fibroblast activity. The systemic effects associated with cytokines release from macrophage include a fever as well as leuco-

cytosis meaning the accumulation of white blood cells circulating within the blood vessels. Cytokines also promote the immigration of immune cells such as neutrophils and monocytes to the inflamed area. When the monocytes move from the blood vessels to the tissues, they become macrophages.

As a consequence of vasodilation and vascular permeability, there will be vascular stasis, which means slowing of the blood in the blood stream with vasodilation and fluid exudation to allow chemical mediators and inflammatory cells to collect and respond to the stimulus.

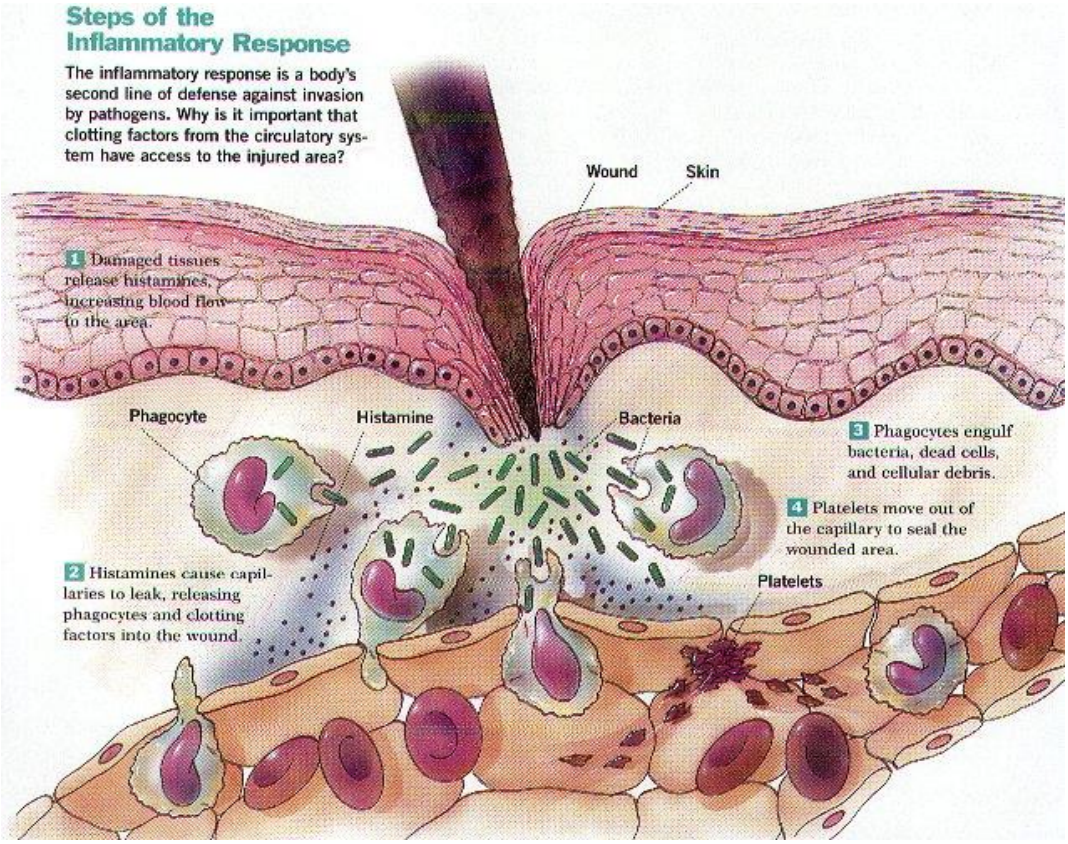


Figure 1: Steps of inflammatory response [8]

Inflammation is normally controlled and self-limited. However, components of the inflammatory process may destroy normal tissue and inappropriate inflammatory

processes (autoimmune reaction) may lead to disorder [8].

Infectious and inflammatory diseases are the most common causes of mortality worldwide [10]. Rational diagnosis of infection and inflammation is invaluable for better outcome and overall costs of the disease management in which radiopharmaceuticals play an important role. Several radiopharmaceuticals have been developed in the last decade for infection and inflammation imaging [11]. Some of them have been used in clinical practice and many others are still in preclinical and clinical studies. Among the ligands for radiopharmaceuticals that used to detect infectious and inflammatory foci is human immunoglobulin G polyclonal antibody.

2.2. Immunioglobulin G

Antibodies are highly specific proteins that are produced by plasma cells of the immune systems in response to pathogenic antigen. The primary function of an antibody is to bind to an antigen and label for its destruction. Antibodies are also called immunoglobulins. There are five primary classes of immunoglobulins, namely IgG, IgM, IgA, IgD and IgE. These are distinguished by the type of heavy chain found in the molecule. IgG molecules have heavy chains known as gamma-chains; IgMs have mu-chains; IgAs have alpha-chains; IgEs have epsilon-chains; and IgDs have delta-chains.

Human immunoglobulin G (IgG) antibody is a monomeric glycoprotein and it consists of two identical heavy polypeptide chains (H) and two identical light polypeptide chains (L) joined via non-covalent interaction and covalent interchain disulfide bonds to form Y-shaped structure [12]. Each chain is divided into variable (V) regions and constant (C) regions. A variable region contains specific types of amino acid sequence that can bind to specific antigen whereas the constant regions usually have constant amino acids sequences [13]. Each L chain consists of one variable domain, VL, and one constant domain, CL. The H chains consist of a variable domain, VH, and three constant domains CH1, CH2 and CH3. Each heavy chain has about twice the number of amino acids and molecular weight (~50.000) as each light chain (~25.000), resulting in a total immunoglobulin monomer molecular weight of approximately 150,000. See Figure 2 below.

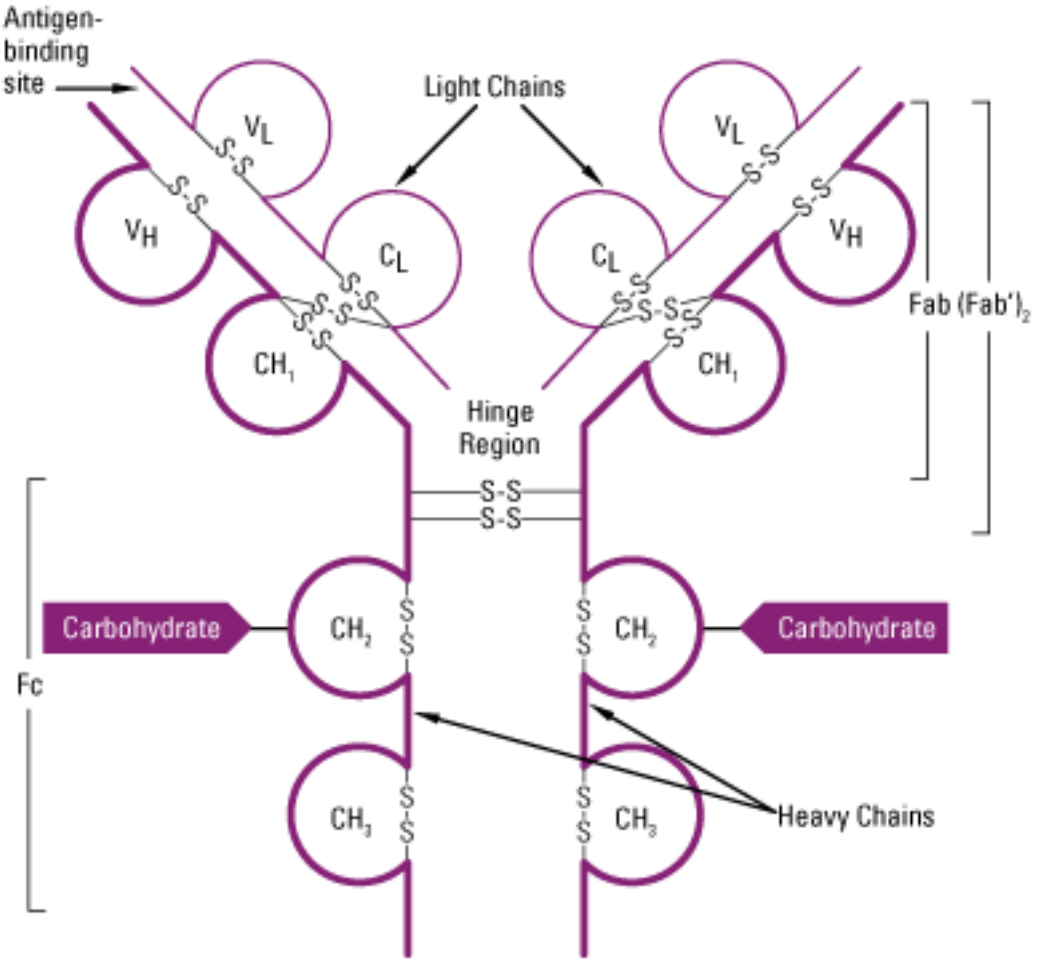


Figure 2: Generalized structure of an immunoglobulin (IgG). (Thermostat scientific)

Human immunoglobulin G (IgG) is the most abundant class of Ig present in human serum. Produced as part of the secondary immune response to an antigen, this class of immunoglobulin constitutes approximately 75% of total serum Ig. IgG is the only class of Ig that can cross the placenta in humans, and it is largely responsible for the protection of a newborn during the first months of life. Because of its relative abundance and excellent specificity toward antigens, IgG is the principle antibody used in immunological research and clinical diagnostics.

IgG antibodies are extracted from donated blood plasma, commercially available and used to treat different immune related abnormalities such as immune deficiencies, auto-immune disorders, and infection. The immunoglobulin G can be labelled with radionuclide directly and indirectly using bifunctional agents and used for imaging of infectious and inflammatory foci. The localization of the radiolabeled immunoglobulin G at the site of infectious and inflammatory foci is due to vascular permeability [14, 15].

2.3. Freeze drying process

Freeze drying is a common method for preparing powdered pharmaceutical products for reconstitution before administration to the patients. The method is suitable for thermolabile drug substances that cannot withstand the heat conditions used in other drying methods. This method has already been used successfully to prepare solid formulations of biological and biotechnological drug products such as immunoglobulins and other proteins [16].

Different substances and formulations respond differently to the freeze drying conditions. As such, every freeze drying process should be tailored and optimized to every particular formulation. An optimized freeze drying protocol is the one that yields the best product characteristics at the least cost.

2.4. Radiopharmaceuticals for Infection and inflammation with the mechanism of accumulation

Several radiopharmaceuticals have been developed to date for the diagnosis of infectious and inflammatory diseases. The ideal radiopharmaceuticals for the imaging of infection and inflammation foci should have high sensitivity for infection/inflammation, differentiate between infection and inflammation, differentiate between acute and chronic infection, no toxicity and/or immunogenic response, minimum radiation burden to the patient and worker, high specificity, rapid clearance from circulation, no uptake in gastrointestinal tract, as well as they should be easy to prepare, have low cost and wide availability [11].

One of the oldest radionuclide to be used for imaging infection and inflammation was ^{67}Ga-citrate. Its localization at the site of infection and inflammation is likely to be due to

Ga^{3+} dissociating from citrate and chelating to blood transferrin. Then this complex extravasates to the site of inflammation due to increased capillary permeability. It also binds to lactoferrin and bacterial siderophores [17] that are abundant at the sites of inflammation and infection. However, it has a limitation like long physical half-life, high energy gamma rays lead to poor quality image and high radiation absorbed dose, non-specific in infectious or sterile inflammations and at last not being available as a generator [18].

Because of the challenges associated with the radionuclide's nuclear characteristics, there was need to explore other radionuclides with better radiation characteristics than gallium-67 (^{67}Ga), which lead to the development of indium labeled radipharmaceuticals. Several ligands can be labelled with indium-111 and used for imaging infection and inflammation foci. Ligands such as human immunoglobulins G polyclonal antibody, monoclonal antibodies, white blood cells, streptavidin-biotin, bacterial chemotactic peptides and liposomes are among the examples. The main advantage of using indium to label with ligands for clinical application is its long half-life (67 hours) which allows imaging beyond 24 hr. post injection.

However, suboptimal photon energies, low resolution images, long time from injection to imaging acquisition in the case of white blood cells [6] and better imaging characteristics of technetium-99m for gamma camera (physical characteristics, availability, cost and lower radiation burden) challenges indium-111's application in the detection of infection and inflammation foci [5].

Technetium-99m is a hugely advantageous radioisotope in infection and inflammation imaging because of the versatile technetium chemistry and its equally important nuclear properties. Since it is primarily available through a commercial GMP compliant generator, it is cheaper than many other radioisotopes used in radiopharmaceuticals such as the cyclotron produced radioisotopes. The 66-hour half-life of the parent molybdenum-99 radionuclide allows for a two-week working life of the generator, thereby allowing for efficient supply logistics [19, 20].

Nowadays ^{99m}Tc-hexamethylpropylene radiolabeled white blood cells (WBCs) is considered as a gold standard method for imaging infection and inflammation. The chemotactic properties of the activated leukocytes form the basis of labeled leukocyte imaging [5]. Unfortunately, the methods have drawbacks. Preparation is time consuming, laborious, needs special facilities, carries a high risk of contamination and is contraindicated in some situations.

Others ligands including antibodies and their fragments, antimicrobial peptides and antibiotics have been labelled with 99m technetium that avoid sophisticated methods of preparation of labeled white blood cells but they are still not devoid of limitation and are nonspecific in differentiating infection and inflammation foci.

Since SPECT technology generally has lower resolution and sensitivity than PET technology. PET radiopharmaceuticals are also given significant interest in the area of infection and inflammation imaging. ^{18}F-fluorodeoxyglucose (^{18}F-FDG), the most commonly used PET radiopharmaceutical has been shown to target sites of infection via increased glucose utilization by infiltrated granulocytes and macrophages due to metabolic requirements. However, this is nonspecific because ^{18}F-FDG is also taken up at nonspecific sites of inflammation as well as sites of tumor [21]. Furthermore, the high cost and limited availability of PET imaging restrict its wide application in infection and inflammation imaging.

3. STATEMENT OF THE PROBLEM

The unavailability of ideal radiopharmaceuticals limits the application of nuclear medicine in the diagnosis of infection and inflammatory disease.

Radiolabeled Human Immunoglobulin G (HIG) may have some clinical utility, but long imaging time, and delayed blood pool clearance.

Commercial kits unavailability limits its application.

4. OBJECTIVE

4.1 General Objective

- To formulate ready use cold kit of human immunoglobulin G (HIG) to be labelled with ^{99m}Tc.

4.2. Specific objectives

- To determine optimum freeze-drying protocols for human immunoglobulin G containing radiopharmaceuticals.
- To compare direct and indirect labeling of human immunoglobulins G with Tc-99m
- To determine the radiochemical purity of obtained radio labeled products

5. EXPERIMENTAL WORK

5.1. Materials

In our study we used the following chemicals:

Human immunoglobulin G (HIG) solutions (50 g/L),

Succinimidyl hydrazi–nonicotinic acid (HYNIC),

Sodium Glucoheptonate,

Sodium Pyrophosphate,

1N HCl,

1N NaOH,

0.1N HCl,

0.1N NaOH,

tricine,

Acetone,

saline (0.9% Sodium Chloride),

Ammonia,

Ethanol (70%vol),

Water for injection,

Distilled water,

0.15M acetate buffer (pH = 6.4),

Phosphate buffered (PBS) saline (0.04 M, pH = 7.4),

Stannous chloride dehydrate

Gel filtration columns (Sephadex, 10mL, 25G and 50G).

The equipment used to obtain our results was:

Freeze Dryer (LBCONCO)

Vacuum pump

Chromatogram scanner,

ITLC paper, Test tubes,

Tubes for ultra-filtration (AMICON)

Centrifuge,

UV-spectrometer,

Filters (Millipore, 0.22 µm),

Mo^{99}/Tc^{99m}generator (ELUMATIC II, IBA Molecular),

Analytical balance (range, 1−20 mg),

Autoclave,

Oven,

Refrigerator,

Laminar airflow cabinet.

5.2. Methods

Purification of human immunoglobulins G (IgG) commercial preparation

The purification of commercial preparation of IgG has been performed by pipetting the required amount of IgG solution into the ultrafiltration tube. Then, the solution was diluted to 2 mL with 0.04 M PBS (pH =7.4). The solution was ultra-filtered by spinning the tube at 5000 rpm for one hour in centrifuge. The ultra-filtration process was repeated three times.

Preparation of human immunoglobulin solutions

Purified human immunoglobulin G (6 mg) was diluted to a concentration of 1 mg/mL using 0.04 M phosphate buffer (pH = 7.4.0) in a 10mL type 1 glass vial. Aliquots (1 mL) of

the resulting solution were dispensed into 6 pre-sterilized pre-weighed 10 mL vials and re-weighed. The procedure was repeated for each freeze drying protocols.

Freeze drying of IgG.

The IgG preparation was freeze-dried using three different protocols as described below in the table. The freeze dryer was turned on and the chamber temperature was monitored until it was 4ºC. The vial was loaded into the freeze dryer with a shelf temperature of 4°C and the process of freeze drying was started. After freeze drying, the vials were capped using rubber caps, blocked with an aluminum ring and stored in a fridge at 4ºC. In Tables 1, 2 and 3 the different freeze drying protocols are reported (Tables 1-3).

Table 1: Three days' lyophilization protocols

Segment	**Freezing temperature**	**RAMP (Speed)**	**Holding Time**
1(Pre-freezing)	Room Temp to 4^0C	0.4^0C/min	30 minutes
2 (Freezing)	4^0C to -45^0C	0.4^0C/min	3 hours
	-45^0C to -15^0C	0.15^0C/min	6 hours
	-15^0C to -45^0C	0.2^0C/min	2 hours
3 (Primary Drying)	-45^0C to -10^0c	0.15^0C/minute	28 hours
4 (Secondary Drying)	-10^0C to 25^0c	0.15^0C/minute	14 hours

Table 2: Two days' lyophilization protocols

Segment	**Freezing temperature**	**RAMP (Speed)**	**Holding Time**
1(Pre-freezing)	Room Temp to 4^0C	1.0^0C/min	30 minutes

2 (Freezing)	4^0C to -45^0C	1.0^0C/min	5 hours
3 (Primary Drying)	-45^0C to -10^0c	0.15^0C/minute	28 hours
4 (Secondary Drying)	-10^0C to 25^0c	0.2^0C/minute	14 hours

Table 3: One day's lyophilization protocols

Segment	**Freezing temperature**	**RAMP (Speed)**	**Holding Time**
1(Freezing)	25^0C to-40^0c	1^0C/minute	5 hours
2 (Primary Drying)	-40^0C to -10^0c	0.12^0C/minute	4 hours
3 (Secondary Drying)	-10^0C to 30^0c	0.08^0C/minute	2 hours

Size exclusion Gel chromatography

Preparation of sephadex gel G25 10mL column

Sephadex G25 (10mg) powder was weighed in a balance to prepare 10 cm *1cm columns. It was submerged in distilled water in a glass beaker and left to swell overnight. The following day, the supernatant was decanted. The gel was packed into four BD® 10mL syringes, washing the columns with 0.04 M phosphate buffer saline (pH=7.4) on every successive addition of gel until the desired level of 10mL was reached. The columns were saturated with 1mL of 0.1% BSA. The columns were each washed with 30mL 0.04 M phosphate buffer (pH =7.4), sealed and stored at 4ºC. The columns were preserved with 3mL of 0.2% sodium azide.

Gel filtration of freeze-dried IgG from different protocols

The freeze-dried product was reconstituted with 1mL saline solution. The reconstitution time was measured using a stopwatch and recorded. The columns were kept at room temperature for one hour and then washed with 30mL 0.04 M phosphate buffer saline (pH=7.4). The number of drops from the column per mL of eluate was determined. The

saline solution of the freeze-dried product was then loaded onto the column and eluted using 0.04 M phosphate buffer saline (pH=7.4). The eluate was collected as 1mL fractions into 10 cuvettes. The absorbance of each cuvette was measured using a UV-spectrometer at 280nm including the blank cuvette containing 1mL 0.04 M phosphate buffer saline (pH=7.4).

Preparation of a lyophilized kit formulation for direct Tc-99m labeling

A kit for direct labeling of human immunoglobulin G with technetium-99m was prepared as follows. Stannous chloride dehydrate (1 mg) and sodium pyrophosphate (or, alternatively, sodium glucoheptonate) (8 mg) were placed in an evacuated vial and then dissolved in 16 mL of 1M HCl. From this vial, 4 mL were withdrawn and added to 1 mL of a purified IgG solution in PBS (5 mg/mL) placed in a vial kept under constant vacuum. From the resulting mixture, 1 mL of solution was dispensed through a 0.22 μm sterile filter to another evacuated vial. Five final vials were obtained, which were stored in a freeze at low temperatures (- 200C) and then freeze-dried.

Preparation of kit for indirect Tc-99m labeling

IgG-HYNIC conjugation

The conjugation of IgG-HYNIC was done as follows: To a solution containing purified human immunoglobulin G (30mg/5ml), 0.5ml of 1M $NaHCO_3$ and 3 molar folds of HYNIC (200μg) dissolved in 100 μl DMSO were added dropwise. The mixture was incubated at 18-25^{0}C for 30 min in the dark. The solution was ultra-filtered with sodium acetate 0.15 M (pH= 6.4) three times and applied to sephadex G-25M PD10 column and eluted with sodium acetate 0.15M (pH= 6.4). The eluent was collected in 1mL fraction in a cuvette and the eluent with high concentration was collected. The concentration of IgG was restored to 4 mg/mL with sodium acetate 0.15M (pH= 6.4). From the resulting mixture, 0.5 mL of solution was dispensed through a 0.22 μm sterile filter to another evacuated vial. Five final vials were obtained, which were stored in a freeze at low temperatures (- 20^{0}C) and then freeze-dried.

Preparation of Sn-Tricine kits

Sn-Tricine kit was prepared by dissolving 400mg of tricin in 25 mL distilled water in evacuated vials. Then, 40 mg of SnCl2.2H2O dissolved in 1 mL of 1N HCl in evacuated vials was added to the tricine solution, the pH was adjusted to 5.3 with 0.8 mL 1 N NaOH. The final volume was adjusted to 40 ml with saline. From the resulting solution, 1 mL was dispensed to five evacuated vials by passing through a 0.22μm filter, which were stored in a freeze at low temperatures (- 200C) and then freeze-dried.

Labeling of freeze-dried kits with Technetium-99m

The labeling of all freeze-dried kits prepared by the described methods were labeled with 99m Technetium using the eluate with containing 74 MBq and 740MBq (specific activity of the eluate).

Sn-tricin kit was reconstituted with 5 ml saline solution. From this solution 50–60 μl was withdrawn and added to a vial containing HYNIC-IgG conjugate. Radiolabeling was performed by adding 20mCi of $^{99m}TcO_4^-$ in 2mL to the mixture and incubated for 30 min at room temperature.

Determination of the radiochemical purity

The total volume of the labeled product was 2 ml. After labeling, we wanted to see the yield of the labeled product and the radiochemical purity.

a. **Direct labeling:**

The radiochemical purity of the radiolabeled IgG was assessed by instant thin-layer chromatography carried out on silica-gel plates (ITLC-SG) using saline as mobile phase. R_f values of the radiolabeled IgG (^{99m}Tc-HIG) and of Tc-99m colloids ranged between 0.0−0.1. Free $^{99m}TcO_4^-$ and ^{99m}Tc-glucoheptonate/pyrophosphate were in the range of

0.9−1.0. Using acetone as a mobile phase, R_f values of ^{99m}Tc-HIG and Tc-99m colloids fell in the interval of 0.0−0.1, whereas R_f values of free $^{99m}TcO_4^-$ and ^{99m}Tc-glucoheptonate/pyrophosphate were 1 and 0.0, respectively. R_f values are summarized in the following Table.

Table 4: Mobile phase, stationary phase and Rf value for radiochemical purity test of the direct labeled kit.

Support	**ITLC-SG**	**ITLC-SG**
Solvent	**Acetone**	**Saline**
R_f ^{99m}Tc-HIG	0.0-0.1	0.0-0.1
R_f $^{99m}TcO_4^-$	1.0	0.9-1.0
R_f ^{99m}Tc-glucoheptonate/pyrophosphate	0.0	0.9-1.0

b. Indirect labeling:

The radiochemical purity of the indirectly labeled human immunoglobulin G (^{99m}Tc-HYNIC-HIG) was analyzed by ITLC-SG using 0.15M citrate buffer (pH = 5.5) as the mobile phase. Rf value of ^{99m}Tc-HYNIC-HIG was $^-$ were in the range of 0.0-0.25. These values for free $^{99m}TcO_4$ and ^{99m}Tc-tricine and Tc-99m colloids were in the ranges of 0.7−1.0 and 0.0−0.25, respectively. Then, ITLC-SG plates saturated with bovine serum albumin (BSA) were employed with EtOH:NH_3:H_2O (2:1:5) as the mobile phase. In this system, Rf values of ^{99m}Tc-HYNIC-HIG free $^{99m}TcO_4^-$ and ^{99m}Tc-tricine were within the interval of 0.7-1.0, whereas Tc-99m colloids ranged between 0.0-0.25. R_f values are summarized in the Table below.

Table 5: Mobile phase, stationary phase and Rf value for radiochemical purity test of the indirectly labeled kit.

Support	**ITLC-SG**	**ITLC-SG saturated with 1%BSA**
Solvent	**0.15 citrate buffer, PH 5.5**	**ETOH: NH_3: H2O (2:1:5)**
R_f ^{99m}Tc-HYNIC-HIG	0.0-0.25	0.7-1.0
R_f $^{99m}TcO_4^-$ and ^{99m}Tc -tricin	0.7-1.0	0.7-1.0
Colloids	0.0-0.25	0.0-0.25

6. RESULTS

Purification of human immunoglobulins G (IgG) commercial preparation

The final concentration in mg/mL of the filtrate was calculated by measuring the absorbance of a sample of the filtered IgG at 280 nm in a UV spectrometer using molar attenuation coefficient of 1.4 L mol^{-1} cm^{-1}.

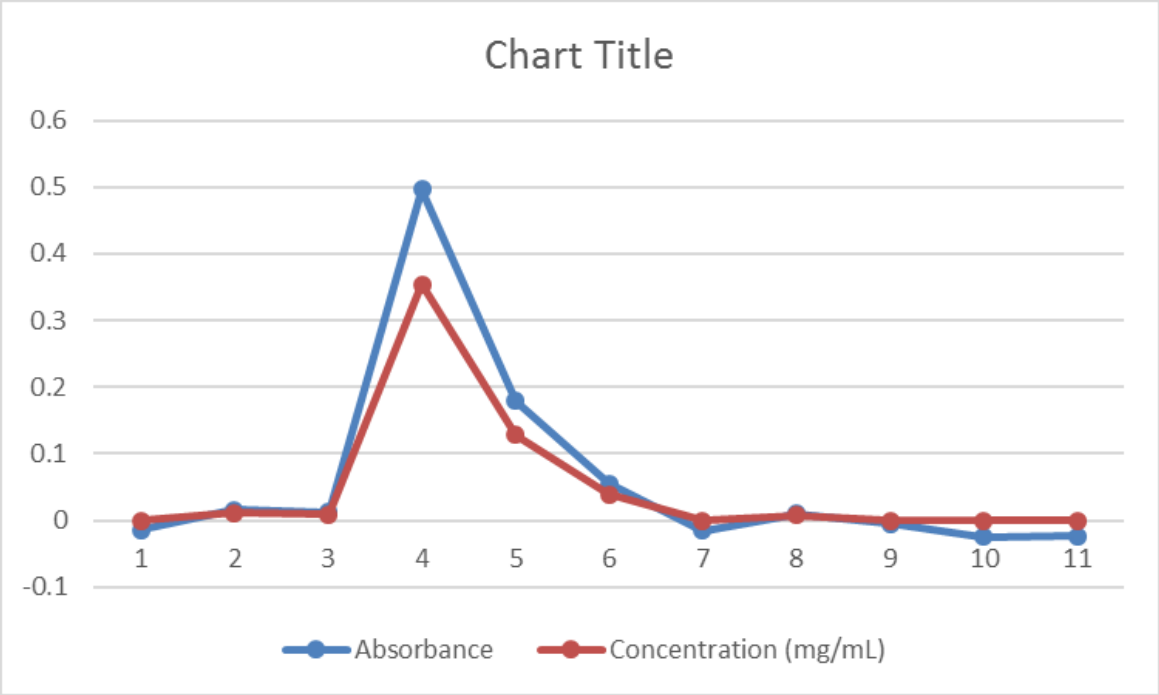


Figure 3: Size exclusion Gel chromatography (SEC) of IgG after purification.

Freeze drying of IgG

The IgG preparation was freeze-dried using three different protocols as described in material and methods. The difference of the three compared protocols was the time of freeze drying that included the time of each segment of the process, speed (RAMP) and holding time.

We used only purified IgG to see which protocol is suitable for the kits containing Immunoglobulin G. The best protocol that we decided to use after the gel filtration of the freeze-dried IgG, dissolved in PBS, with the pH 7.4 (figures) was the two days' protocol.

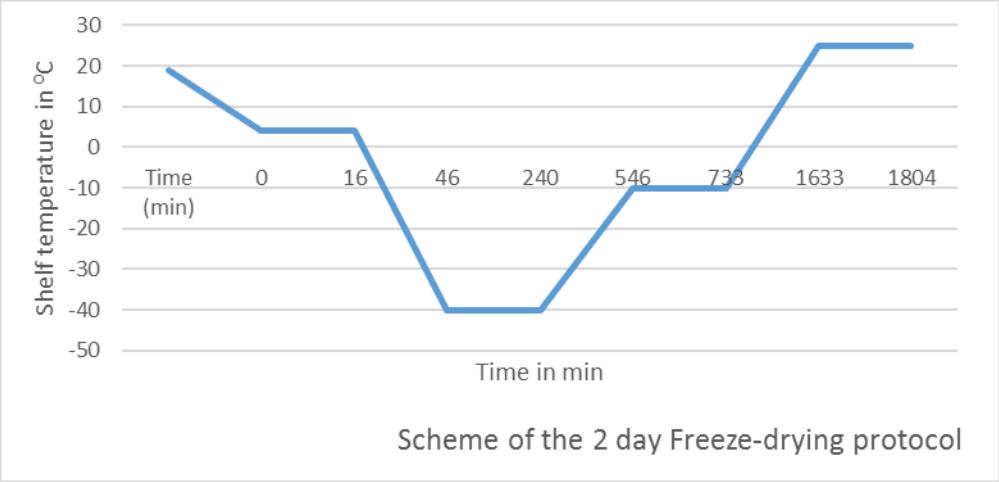


Figure 4: The protocol for freeze drying providing the best results – two days's protocol

Effect of the process of freeze drying of human immunoglobulin

We used all three protocols described in the material and methods and performed a comparison.

a. Weight loss determination after

The vials contacting IgG were weighed before and after freeze drying. The net loss is determined by subtracting the weight of the vials after freeze drying from the weight of vials before freeze drying.

Table 6: The weight loss of 1 mg/ml IgG after two days' protocols lyophilization in open vials

Vials	**Weight of vial and 1mg/ml IgG**		**Net loss**
	Before freeze drying	**After freeze drying**	
1	10.708g	9.715g	0.993g
2	10.662g	9.668g	0.994g
3	10.692g	9.700g	0.992g
4	10.607g	9.614g	0.993g
5	10.868g	9.871g	0.997g
6	10.765g	9.771g	0.994g
7 (1.5ml)	11.245g	9.821g	1.424g

Table 7: The weight loss of 1mg/ml IgG after One day's protocols lyophilization in open vials

Vials	**Weight of vial and 1mg/ml IgG**		**Net loss**
	Before freeze drying	**After freeze drying**	
1	18.056g	17.066g	0.990g
2	18.236g	17.229g	1.007g
3	17.616g	16.622g	0.994g
4	17.408g	16.404g	1.004g
5	18.146g	17.147g	0.999g
6	18.176g	17.255g	0.921g
7 (1.5ml)	18.251g	17.172g	1.079g

We compared the weight loss from two protocols and we found that t critical (2.179) is greater than t calculated (0.8762). The weight loss from two days' protocols is not statistically different from the weight loss from one-day freeze drying protocol.

b. Velocity of the reconstruction for freeze-dried product

The time taken to completely dissolve the constituents of the lyophilized products was recorded by a stopwatch in seconds. The stopwatch was started when the solvent was introduced into the product and stopped when the complete dissolution was noticed by naked eye.

Table 8: Time of reconstitution for freeze-dried IgG

Sample	Time of reconstruction (s)
Three days' closed	25-48
Two days' closed	31-45
Two days' open	55-60
One-day' open	29-46

c. Gel filtration

We performed Gel filtration for each freeze-dried products. The antibody concentration in each cuvette was determined by using the absorbance from UV-visible spectrometer using molar attenuation coefficient of 1.4 L mol^{-1} cm^{-1} against a blank cuvette containing 1mL 0.04 M phosphate buffer saline (pH=7.4). The results were presented in a table and in a line graph spectrum.

Cuvette number	Absorbance	Concentration (mg/mL)
Voide volume(1)	0.007	0.05
2	0.065	0.046428571
3	0.012	0.008571429
4	0.338	0.241428571
5	0.554	0.395714286
6	0.194	0.138571429
7	0.07	0.05
8	0.069	0.049285714
9	-0.029	0
10	0.022	0.015714286
11	0.035	0.025

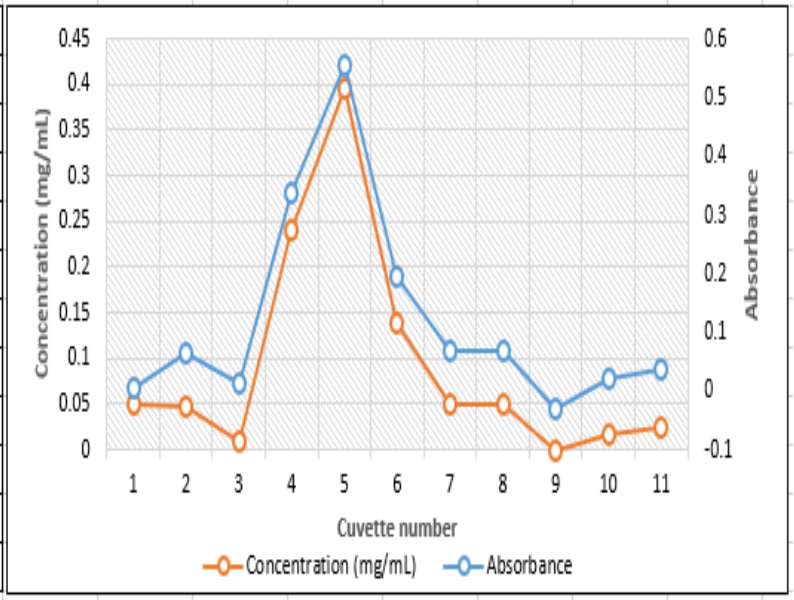


Figure 5: Size exclusion Gel chromatography (SEC) of IgG 3-days' freeze drying protocol using SEPHADEX Gel G25 Column

Cuvette number	Absorbance	Concentration (mg/mL)
Voide volume(1)	0.011	0.007857143
2	-0.002	-0.001428571
3	0.016	0.011428571
4	0.319	0.227857143
5	0.751	0.536428571
6	0.095	0.067857143
7	0.02	0.014285714
8	0	0
9	0.044	0.031428571
10	0.006	0.004285714

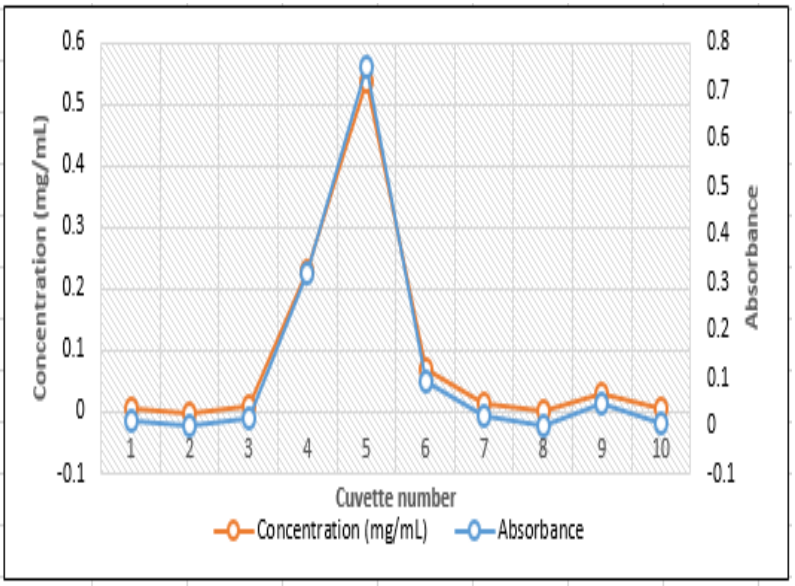


Figure 6: Size exclusion Gel chromatography (SEC) of IgG 2-days' freeze drying protocol using SEPHADEX Gel G25 Column

Cuvette number	Absorbance	Concentration (mg/mL)
Void volume (1)	0.023	0.016428571
2	0.095	0.067857143
3	0.106	0.075714286
4	0.474	0.677
5	0.5	0.715
6	0.189	0.135
7	-0.045	-0.032142857
8	0.104	0.074285714
9	0.128	0.091428571
10	0.143	0.102142857

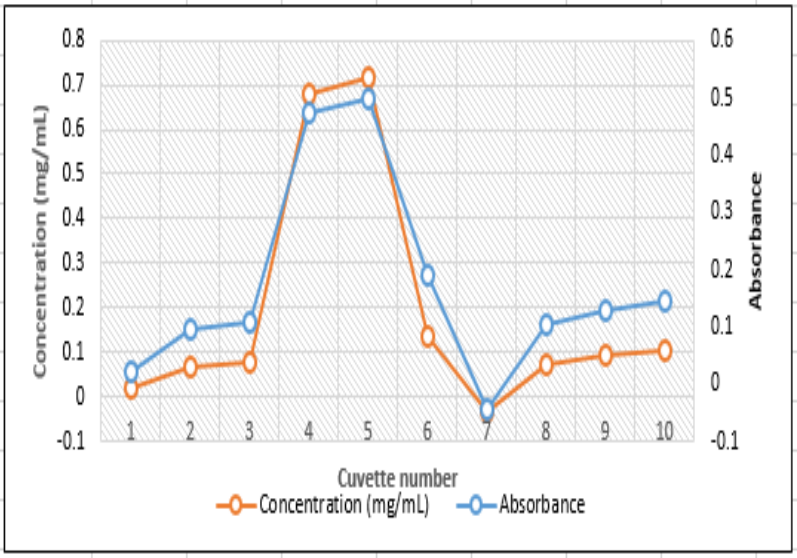


Figure 7: Size exclusion Gel chromatography (SEC) of IgG 1-day' freeze drying protocol using SEPHADEX Gel G25 Column

Preparation of a kit for direct labeling

Kits for direct labeling contain purified human immunoglobulin G polyclonal antibody, stannous chloride dehydrate, and weak ligand sodium pyrophosphate or sodium gluconate. We freeze dried and checked for radiochemical purity.

Preparation of a kit for indirect labeling

The kit for indirect labeling was prepared using the described method. During the preparation, we used HYNIC as a bifunctional chelating agent. We performed gel filtration chromatography to separate the conjugate from unlabeled IgG and HYNIC. See the graph below:

Conjugation of IgG -HYNIC

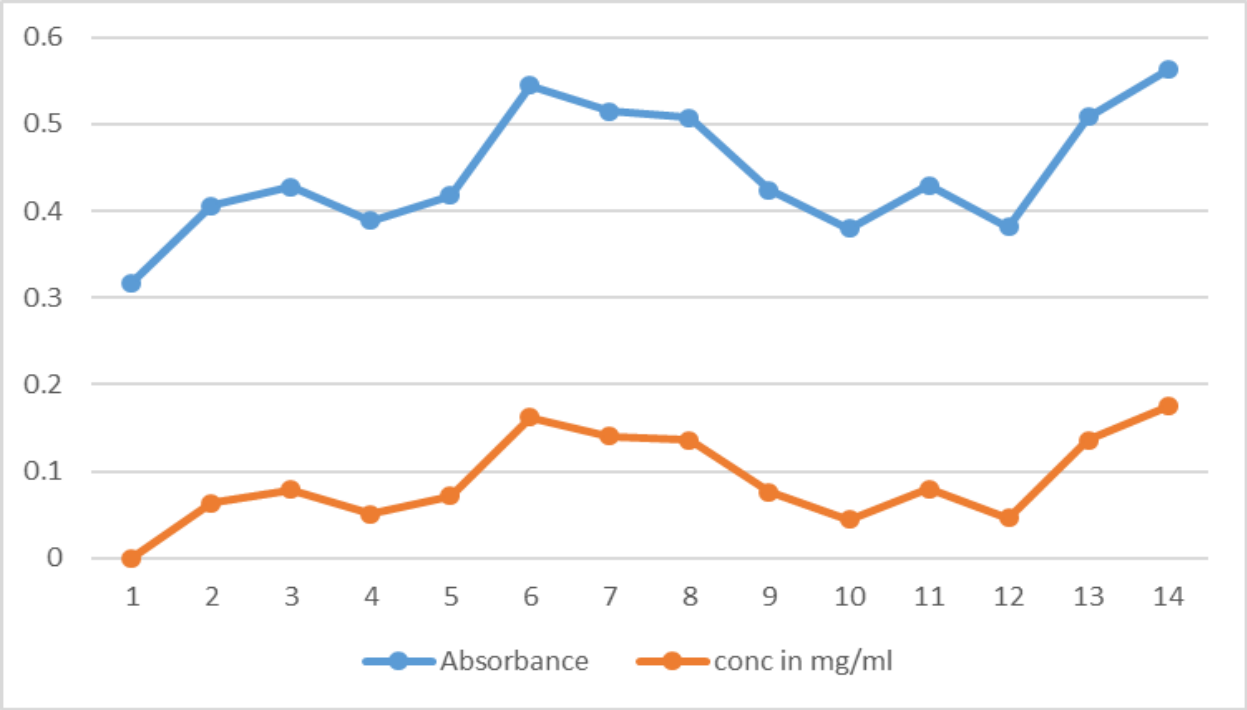


Figure 8: SEC of IgG conjugated HYNIC before ultrafiltration

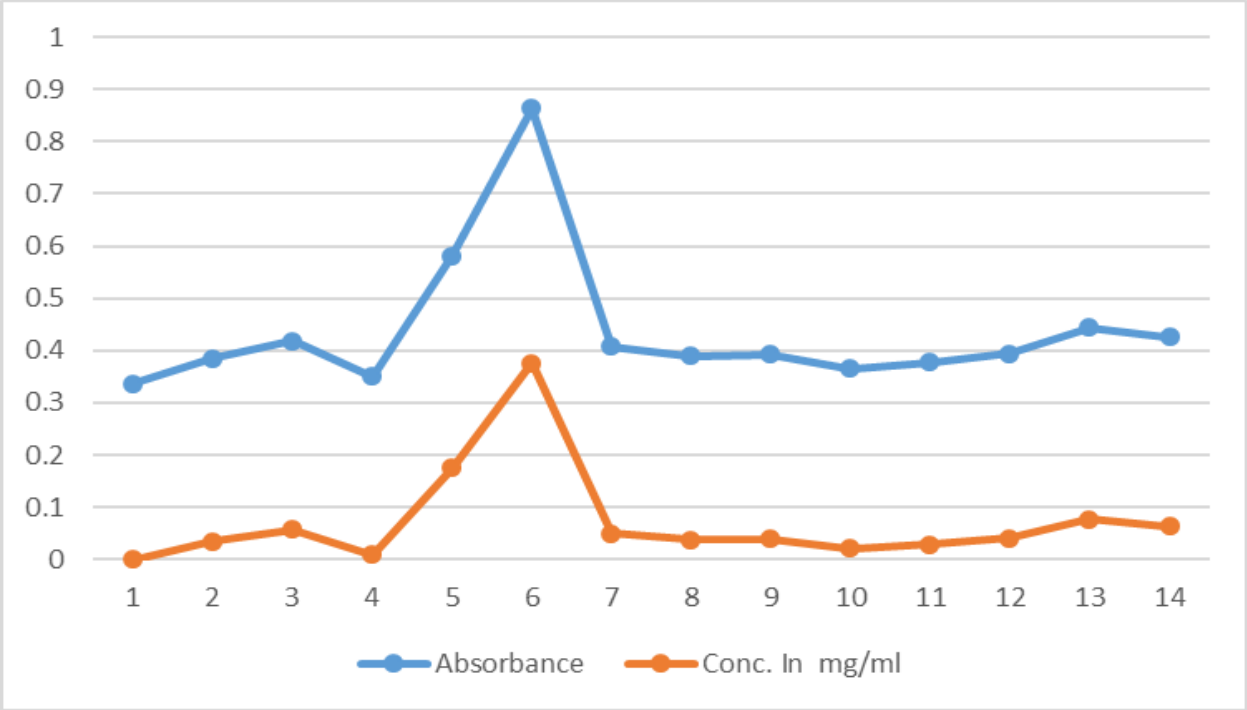


Figure 9: SEC of IgG conjugated HYNIC after ultrafiltration

Preparation of Sn-Tricin kits

Freeze drying of IgG containing kit

For our kits prepared as explained in our methods and after testing the proposed methods of freeze drying - one day', two days' and three days' protocols. We decided to use the two days' protocol (Figure 4) for the reason that it provided us with more acceptable results.

Labeling of freeze-dried kits with Technetium-99m

The labeling of all freeze-dried kits prepared by the described methods was done with 99m Technetium using the eluate with containing 74 MBq and 740 MBq (specific activity of the eluate 5.55 GBq/ml).

The Sn-tricin kit was reconstituted with 5 ml saline solution. From this solution 50–60 µl was withdrawn and added to a vial containing HYNIC-IgG conjugate. Radiolabeling was performed by adding 20mCi of $^{99m}TcO_4^-$ in 2mL to the mixture and incubated for 30 min at room temperature.

Determination of the radiochemical purity

The total volume of the labeled product was 2 ml. After labeling, we wanted to see the yield of the labeled product and radiochemical purity. The radiochemical purity was realized 30 minutes after the labeling and 60 minutes after the labeling using the same solvents and material.

Direct labeling:

Radiochemical purity of the radiolabeled IgG was assessed by instant thin-layer chromatography as described in Material and methods.

Indirect labeling:

Radiochemical purity of the indirectly labeled human immunoglobulin G (^{99m}Tc-HYNIC-HIG) as described in Material and methods.

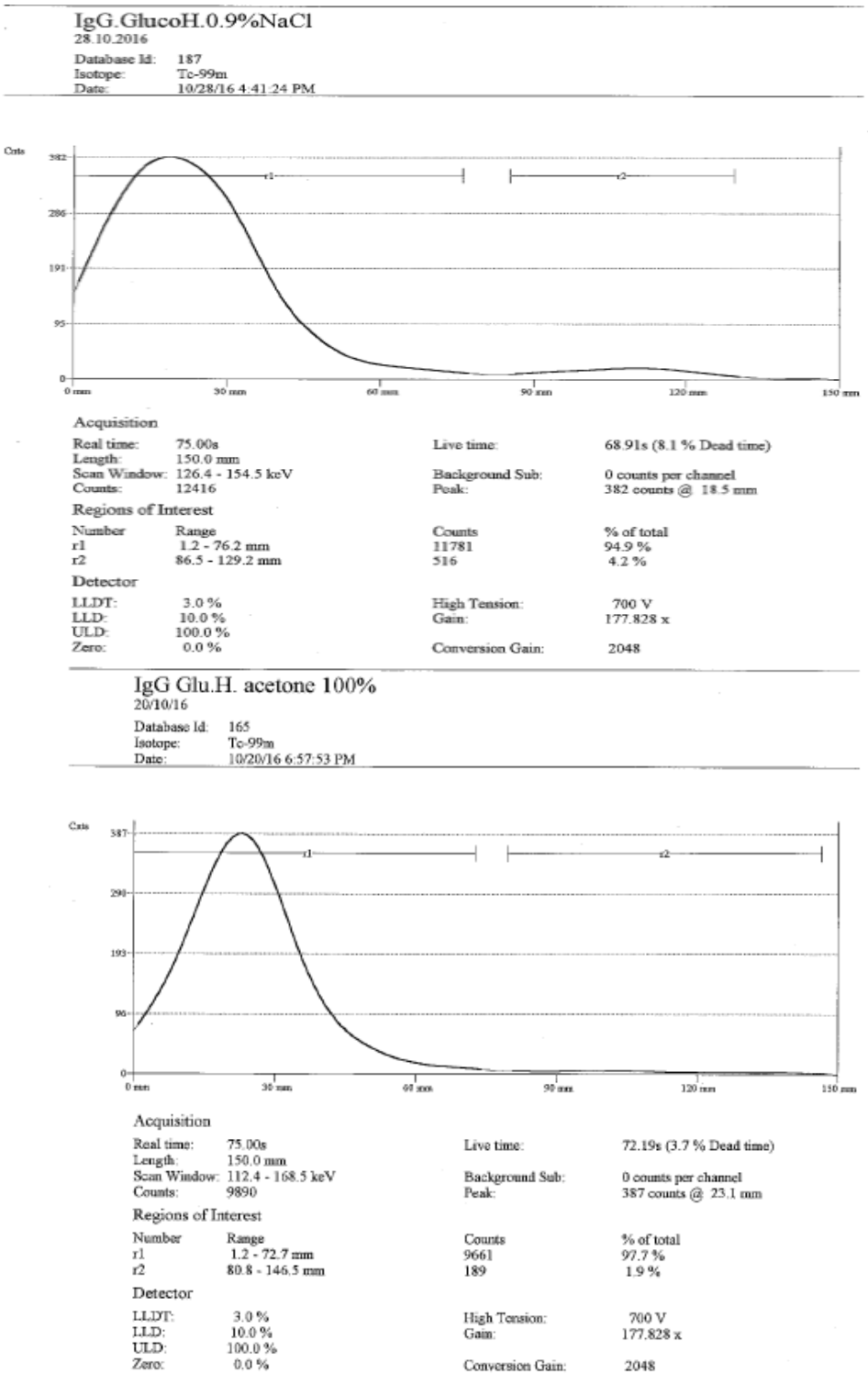
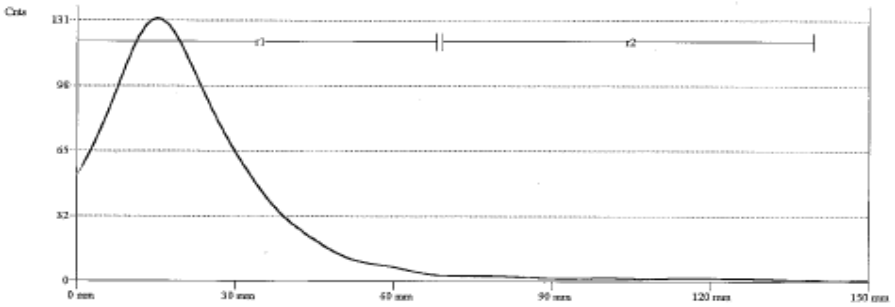


Figure 10: Radiochromatogram of ITLC -SG as the stationary phase, acetone as the mobile phase and saline as the mobile phase for Glucoheptonate containing a directly labeled kit.

IgG.HYNIC.ACETONE 100%
20.10.16

Database Id: 168
Isotope: Tc-99m
Date: 10/20/16 7:27:12 PM



Acquisition

Real time:	75.00s	Live time:	73.97s (1.4 % Dead time)
Length:	150.0 mm		
Scan Window:	112.4 - 168.5 keV	Background Sub:	0 counts per channel
Counts:	3381	Peak:	131 counts @ 15.0 mm

Regions of Interest

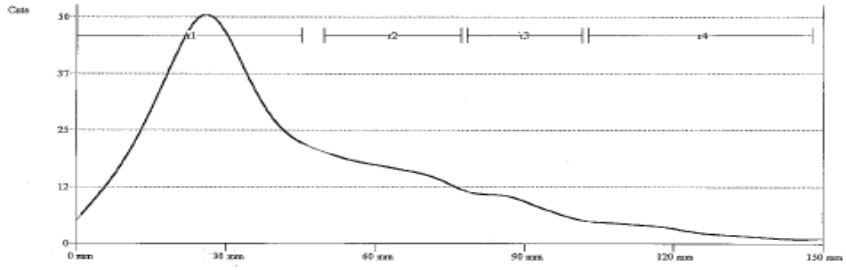
Number	Range	Counts	% of total
r1	1.2 - 68.1 mm	3281	97.0 %
r2	70.4 - 139.6 mm	89	2.6 %

Detector

LLDT:	3.0 %	High Tension:	700 V
LLD:	10.0 %	Gain:	177.828 x
ULD:	100.0 %		
Zero:	0.0 %	Conversion Gain:	2048

IgG-HYNIC Saline 0.9%
20/10/16

Database Id: 169
Isotope: Tc-99m
Date: 10/20/16 7:41:09 PM



Acquisition

Real time:	75.00s	Live time:	73.98s (1.4 % Dead time)
Length:	150.0 mm		
Scan Window:	126.4 - 154.5 keV	Background Sub:	0 counts per channel
Counts:	1969	Peak:	50 counts @ 25.4 mm

Regions of Interest

Number	Range	Counts	% of total
r1	1.2 - 45.0 mm	1184	60.1 %
r2	50.8 - 77.3 mm	398	20.2 %
r3	79.6 - 101.5 mm	179	9.1 %
r4	103.8 - 147.7 mm	104	5.3 %

Detector

LLDT:	3.0 %	High Tension:	700 V
LLD:	10.0 %	Gain:	177.828 x
ULD:	100.0 %		
Zero:	0.0 %	Conversion Gain:	2048

Figure 11. Radiochemical purity of IgG HYNIC tricine performed by ITLC

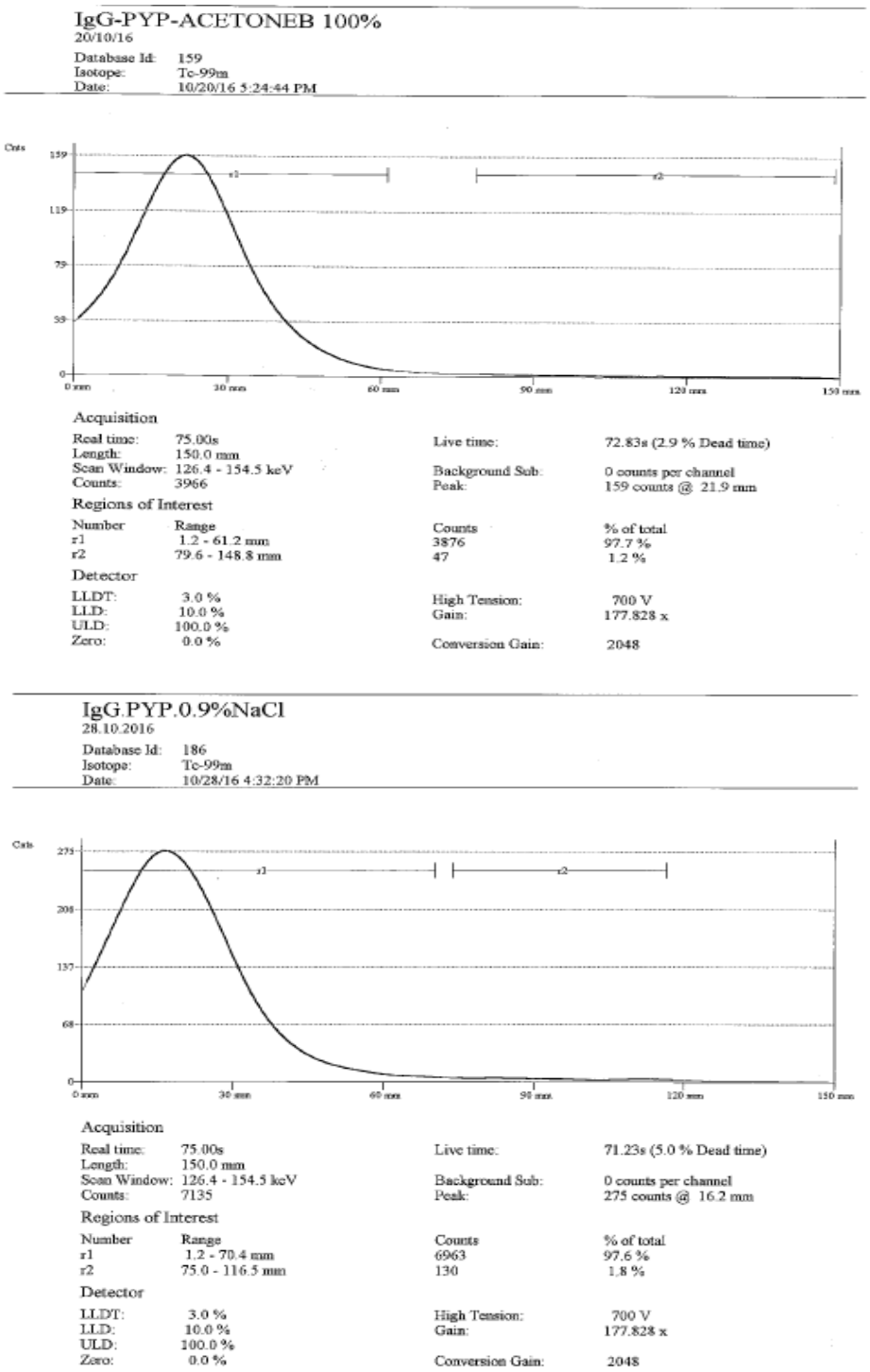


Figure 11: Radiochromatogram of ITLC -SG as the stationary phase, acetone as the mobile phase and saline as the mobile phase for Pyrophosphate containing a directly labeled kit.

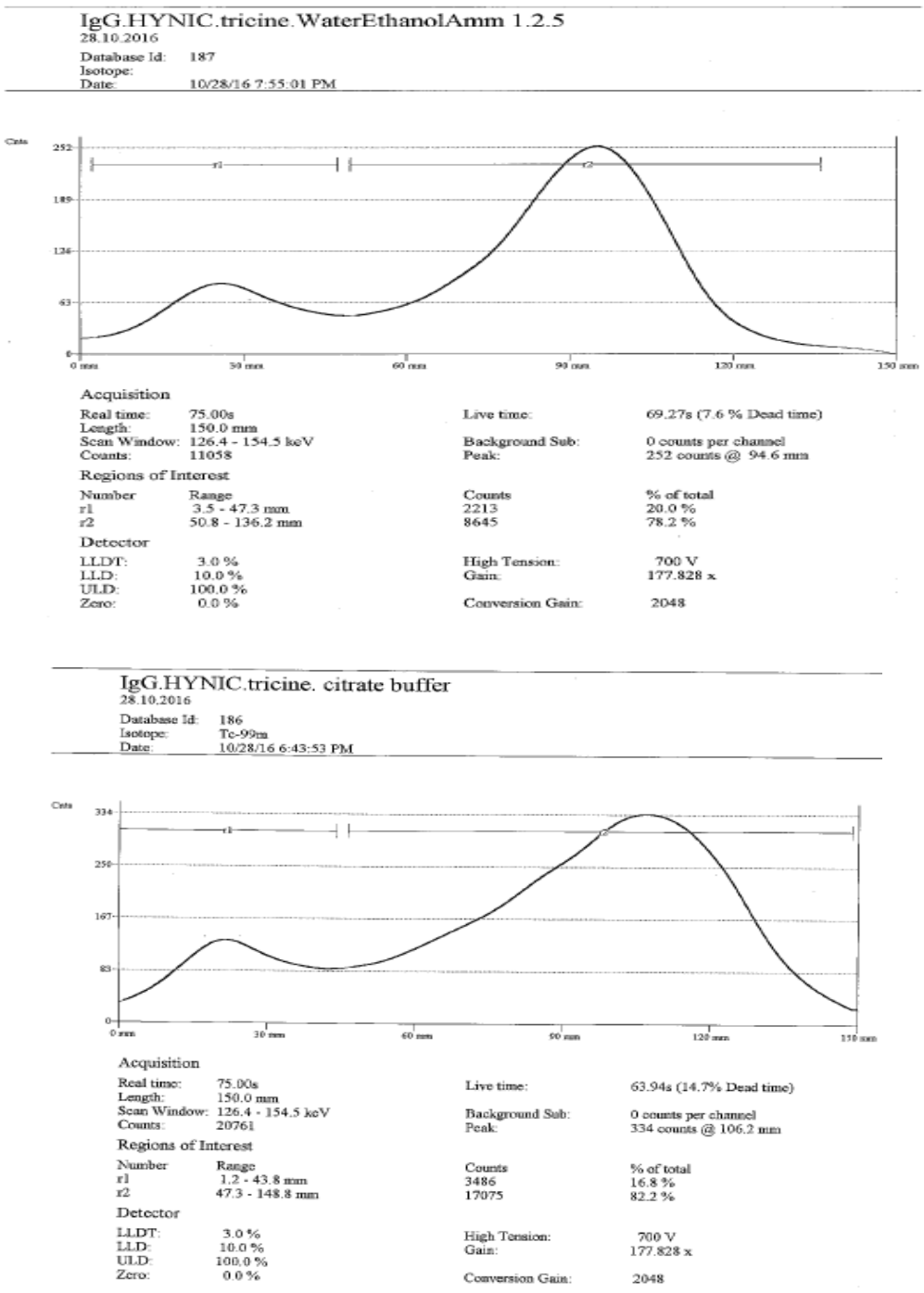


Figure 12: Radiochromatogram of ITLC -SG saturated with 1% BSA as the stationary phase, 0.15 citrate buffer, (pH =5.5) as the mobile phase and ETOH: NH3: H2O (2:1:5) as the mobile phase for an indirectly labeled kit

7. DISCUSSION

Human immunoglobulin G solutions as pharmaceutical products have already been available on the market for clinical practice. The commercial human immunoglobulin G preparation contains different additives and preservatives apart from the human immunoglobulin G antibody. These additives and preservatives should be removed from the preparation to use the antibody for the preparation of the radiopharmaceuticals as these components may interfere with conjugation and radiolabeling of the IgG preparation. A different purification technique of antibody can be used to remove this additive from the human immunoglobulin antibody solution such as dialysis, gel filtration chromatography, ion exchange chromatography and ultrafiltration. We found that the ultrafiltration purification technique is simple, easily available and cheap. We performed ultrafiltration until the antibody concentration of the filtrate was close to zero as measured in the UV-spectrometer at 280nm.

Human immunoglobulin G containing radiopharmaceuticals kits in our preparation are usually in a concentration of 1mg/mL for direct labeling and 2mg/mL for indirect labeling. To compare the effects of different freeze drying protocols, we used a concentration of 1mg/mL IgG solution.

The stability of antibody containing radiopharmaceuticals in solution forms is usually low with long time storage. The development of stable, easily stored and shipped IgG containing radiopharmaceuticals requires the use of the freeze drying technology. However, the lyophilization process is costly, time consuming and may produce damage to the antibody. It is important to determine the optimal lyophilization protocols for our IgG preparation.

The freeze drying of human immunoglobulin G for radiopharmaceutical kits for technetium-99m radiolabeling has already been done successfully [15]. The aim of our study was to compare the effectiveness of the reported freeze drying protocols with modified protocols so as to make recommendations on their suitability. The products were freeze dried using the IAEA recommended one-day protocol, two-day protocol and a three-day protocol.

We performed a comparison of lyophilization protocols using gel filtration chromatogram. Gel filtration is a popular method of analyzing liquid mixtures of substances that differ by their molecular masses. The gel is a bed with pores of defined sizes. Since they cannot pass through the pores, larger molecular mass substances readily navigate between the pores as they flow down and out of the bed as they elute earliest with minimal impedance. The substances with smaller masses, however, are small enough to go through most of the pores on their way down the bed. The motion of the smaller molecular mass substances is more retarded by the column packing, making them to elute later than the substances with higher molecular masses [22].

By collecting sequential fractions of the eluate and determination of the concentration of the substances in every eluate, it is possible to analyze the composition of the sample solution by molecular masses of the components. This method has been used previously in studies similar to ours to assess the amounts of immunoglobulin fractions in immunoglobulin sample solutions [23].

In our study, it was important to analyze the immunoglobulin G solutions obtained from different freeze drying protocols so as to determine the concentration of immunoglobulin fragments in the mixtures. An inferior freeze drying protocol is expected to yield a freeze-dried product upon reconstitution of which it produces a solution with high amounts of immunoglobulin fragments. From our results above, less immunoglobulin fragmentation was observed with the sample solution from the two-day protocol.

Human immunoglobulin G ploYclonal antibodies have been labeled with technetium in the two methods: direct and indirect methods. Direct labeling methods require reduction of the antibody disulfide bond, so as to generate the thiosulfate functional groups where reduced technetium can bind to the antibody. We used the stannous chloride dehydrate as a reducing agent to reduce both the disulfide bond and the technetium. Stannous chloride dehydrate is widely available and demonstrated that it is the preferred reducing agent for technetium radiopharmaceuticals (Spies H 2007). The drawback with the direct method is that fragmentation of the antibody may occur and also technetium is unstable towards trans chelation.

On the other hand, the indirect method of technetium labeling of human immunoglobulin G employs the use of bifunctional agents which can attach very easily to the amino group of the antibody and the technetium. In our case, we use HYNIC bifunctional agents since-HYNIC has been widely used as a bifunctional chelating agent for technetium -99m labeling with peptides and protein and it also forms strong bonds between technetium and the antibody. Tricine is important as a co-ligand to stabilize the Tc-HYNIC binding and the kit also contains stannous ion for reducing the technetium.

Ultrafiltration and Gel filtration techniques were used for the purification of the IgG-HYNIC conjugate to remove any excess ligands, damaged and fragmented IgG antibody during conjugation, as shown in Figures 8 and 9. We first did size exclusion chromatography of the conjugated antibody using sephadex G-25M column and we observed that the graph of the concentration of antibody is not consistent with a multiple pick. Then we performed ultrafiltration on the conjugated product and after that we performed size exclusion gel chromatography and obtained a defined pick. We collected a cuvette with high concentration and diluted with phosphate buffer saline 0.04 M (pH=7.4) to the concentration of 4mg/ml, then 0.5 ml of the final solution was dispensed to evacuated vials passing through 0.22μm filters to be lyophilized using the two-days' protocols. As we revealed, the two-days' protocol is a suitable lyophilization protocol for our products.

We used the same activity to label kits for direct labeling and indirect labeling. The technetium-99m is obtained from IBA, molecular generators 99Mo/99mTc. The necessary quality control test was performed on technetium eluent before labeling. We used an activity of 74MBq and 740MBq measured in the dose calibrators for labeling the kit. For direct methods, we used the onestep labeling methods; the activity in a volume of 2 mL was withdrawn from eluent vials and added to the kits. For indirect labeling, Sn-tricin kits were diluted with 5mL saline, then the 50-60 ul was withdrawn and added to the IgG HYNIC conjugate. Then technetium activity in 2mL was added. The solution was clear and free from particulate matter.

One of the main quality control tests that should be performed on radiopharmaceuticals is radiochemical purity. We checked the radiochemical purity of our preparation using planar chromatography, which is time saving and easy to perform. ITLC SG as stationary

phase, by applying a drop of sample from the kit for direct labeling we developed two chromatogram strips, one for the acetone mobile phase and the other for the saline mobile phase.

We were able to separate the radiochemical impurities, free pertechnetate and 99mTc-glucoheptonate/pyrophosphate respectively. The overall labeling efficiency was greater than 90%. For indirect labeling, using ITLC SG as stationary phase and 0.15 citrate buffer, PH 5.5 as a mobile phase, we able to separate 99mTcO4- and 99mTc -tricin from 99mTc-HYNIC-HIG, 99mTc-colloids. To determine the Tc- colloids content, we used ITLC SG saturated with bovine serum albumin as a stationary phase and ETOH: NH3: H2O (2:1:5) as a mobile phase. The overall labeling efficiency was low. We observed a slow movement ETOH: NH3: H2O (2:1:5) on ITLC-SG saturated with BSA.

8. CONCLUSION

Purification of the commercial IgG preparation before any subsequent procedure in the preparation of radiopharmaceuticals kits is important to remove any additives that may interfere with the labeling.

From the study, different freeze drying protocols can be applied to freeze dry IgG but the two-days' protocols give less antibody fragments than other protocols.

Human immunoglobulin G can be labeled directly or indirectly using a bifunctional agent. Direct labeling of the prepared kit yields better labeling efficiency.

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