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ABSTRACT

This paper describes a method for labelling immunoglobulin with ^{99m}Tc that reduces a small number of protein dissulfide bonds and facilitates labelling with high yields (Mather and Ellison,1990) Simple fast and reliable analytical techniques were developed to assess radiolabelling efficiency and ^{99m}Tc-hIgG stability The choice of buffer pH was essential for obtaining a radiolabelling yield ≥ 99% Very good ^{99m}Tc -hIgG stability was obtained with hIgG/MDP mixture (locally prepared) in the form of a lyophilized kit, which makes it possible candidate for scintigraphy use

AVANÇOS NA MARCAÇÃO DE ANTICORPOS COM ^{99m}Tc

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RESUMO

Descrevemos um metodo para marcação de imunoglobulina com ^{99m}Tc que reduz as pontes dissulfídicas da proteína permitindo alta eficiencia de marcação (Mather e Ellison, 1990) Desenvolvemos metodo simples, rapido e seguro para a avaliação da eficiência de marcação e estabilidade da preparação [^{99m}Tc-hIgG] Para o alcance de uma eficiência de marcação alta e importante a escolha do tampão e pH Obtivemos excelente estabilidade com a mistura hIgG (reduzida)/MDP preparada em nosso laboratorio em forma de kit liofilizado apropriado a um possível uso cintilografico

INTRODUCTION

The use of radiolabelled monoclonal antibodies (MAbs) for detection of cancer has been the focus of a considerable research effort Colorectal cancer has been the tumor type most frequently studied with radiolabelled antibodies The utility of radiolabelled antibodies in the radioimmunodetection of cancer has been established with ^{131}I, ^{111}In and ^{123}I (Goldenberg et al,1978 and Colcher et al,1987)

Studies on MAbs labelling were started in our laboratory, some years ago, with the preparation of radioiodinated (^{131}I) monoclonal antibody 4C11 belonging to IgG_{2a} subclass from mouse ascitis donated by Ludwig Institute/Brazil Iodination was performed by Iodogen Method (Fraker and Speck,1978) Although iodine-131 is probably an adequate label for some radioimmunodetection, it also has several characteristics such as dehalogenation *in vivo*, emission of medium-energy gamma rays, relative long half-life, maximum beta range of about 200 cell diameters, which limit its utility for labelling MAbs This has come about with the application straightforward, *in vivo* stable,methods monoclonal antibodies with ^{99m}Tc This radionuclide has ideal nuclear properties for gamma cameras and is readily available from generator at minimal cost Indeed, successful clinical application of comercial monoclonal antibody imaging products will be dependent on the development of simple, inexpensive methods to label antibody with ^{99m}Tc

Significant research over past decade has focused on ^{99m}Tc labelling of proteins by bonding the radionuclide to reactive sulfides Two mechanisms have been employed to provide reactive sulfides in proteins that are to be labelled with technetium directly or through the use of a bifunctional chelating agent Direct labelling methods are generally preferred at present The direct labelling of sulphydryl residues on antibody by Technetium has been extensively explored (Park et al,1985) Several variations of the direct labelling method have been reported These variations primarily involve alternate methods of antibody reduction Pioneering work towards a simple and efficient incorporation rate labelling technique was done by Schwarz and Steinstraesser,1987 The method of Schwarz using 2-dimercaptoehanol as

a reducing agent to expose the -SH groups for binding reduced ^{99m}Tc, lead the way to introduction of a range of agents with similar actions Mather and Ellison,1990 have studied the reduction of dissulfide bridges using 2-mercaptoethanol and found that increased reduction was paralled by increased labelling efficiency The reduction dissulfides is a necessary initial step in ^{99m}Tc labelling of antibodies A major advantage of the direct labelling method is that it can readily be reduced to a one step labelling process which is beneficial for making a marketable radiopharmaceutical preparation kit

The purpose of this study is to examine a convenient system that can be used to radiolabelled antibodies which is rapid, simple, efficient and reproducible, and which can be accomplished in radiopharmaceutical laboratories Human immunoglobulin (hIgG) Sandoz Inc is used as model to evaluate the radiolabelling with ^{99m}Tc and quality control procedures as a preliminary assessment for their specific application in scintigraphy of focal inflamatory lesions (Thakur et al,1991) Modified Schwarz s direct labelling technique by Mather and Ellison, 1990 is adopted for labelling studies The technique employs 2-mercapto ethanol (2-ME) for reducing interheavy chain dissulfide bridges at hinge region, the presumed high affinity site for technetium binding Reduced ^{99m}Tc probably at oxidation state 5 generated by sufficiently small amount bone -scanning kit, methylene diphosphonate MDP-SnII, chelated by 2-ME-reduced sulphydryl groups to form complex It is likely that the specified amount MDP-SnII would allow technetium reduction to the oxidation state 5 The application of small amount of MDP-SnII for successful antibody labelling has clearly indicated the need of high quality MDP-SnII kit for reproducible labelling out comes For this purpose an MDP bone scanning (Amersham) have been used It is likely that alternative sources of MDP can be used successfully (Mather and Ellison, 1990)

This paper describes, based on Mather s method, our experimental conditions for labelling immunoglobulin (hIgG) with ^{99m}Tc The reduced hIgG preparations were labelling using two radiopharmaceutical kits (Amerscan MDP Amersham) containing 5 mg medronate (MDP), 0 34 mg stannous fluoride (SnF_2), and 2 mg p-amino benzoic acid, MDP-IPEN/Brazil containing 5 mg medronic acid (MDP), 0 75 mg stannous chloride

($SnCl_2$ $2H_2O$), and also the same Amersham kit formulation locally prepared Since the first experiments demonstrated similar results the MDP like Amersham kit formulation prepared in our research laboratory was selected for hIgG labelling

MATERIAL AND METHODS

Protocol for labelling antibody with Technetium-99m

Lyophilized immunoglobulin hIgG (Sandoz Inc) was dissolved in sterile saline Aliquots containing 10 mg were prepared and stored at 20 C until to be used

This study does not involve human administration of ^{99m}Tc-hIgG Nevertheless some effort was devoted to preparing the ^{99m}Tc-hIgG under sterile and apyrogenic conditions to move one step closer to eventual human administration

Antibody reduction

Sufficient 2-mercaptoethanol was added to a stirred solution of antibody provide a molar ratio of 1000 1

The mixture was incubated at room temperature for 30 minutes

The reduced antibody was purified by gel filtration on a 20 ml Sephadex G-50 fine column and eluted using cold N_2-purged neutral phosphate buffered saline as the mobile phase

Fractions of approximately 3 0 ml were collected

The concentration of protein was determined by optical density at 280 nm on a UV spectrophotometer (absorbance of 1 mg/ml solution of IgG = 1 4 cm light path)

The tubes containing protein with concentrations greater than 1 mg/ml were pooled

Aliquots (0 5 mg) were dispensed into sterile vials frozen immediately at -20°C and stored ready for use

Radiolabelling

Ten vials containing 0 5 mg of reduced antibody and 40 µl of MDP solutions, locally prepared, containing 40 µg of MDP and 2 72µg of SnF_2 were prepared in the form of a lyophilized kit It is useful to stability studies to identify potential improvements that could be investigated For example what would be the effect of adding the MDP solution immediately antibody reduction and prior or during the storage

Radiolabelling of the lyophilized kit

Approximately 1 mCi of ^{99m}Tc eluted from a $^{99}Mo/^{99m}Tc$ generator (Medgenix,Belgic) in the form of pertechnetate [$^{99m}TcO_4^-$] (IPEN-TEC) in 1 ml normal saline was added to each kit (reduced hIgG/MDP mixture) to be radiolabelled The radiolabelling reaction was allowed 10 minutes Labelling efficiency was measured by miniature chromatographic system (Kazikiewcz et al,1987)

Quality assurance

Radiochemical purity and label stability of the preparation were measured by miniaturized chromatographic procedures at the time intervals of the first and 20th days post kit preparation The stability of the labelling was tested *in vitro* by determining the % activity associated with the protein as a function of time

The miniature chromatographic system

This system was elaborated to determine the labelling efficiency the radiochemical purity and *in vitro* stability of the preparation

The miniaturized chromatographic procedures was performed using Whatmann 3MM paper (1 cmx6 5 cm) as solid phase and 0 9% saline as mobile-phase The paper was spotted at 1 cm from the bottom The strips were placed in a vial containing approximately 1 ml of 0 9% sa

line The chromatograms was developed for a distance of 5 cm The elapsed developing time was approximately 10 minutes The advantage of this method is that the radiochromatographic systems are chosen such that in one the impurities move with the solvent front (Rf = 0 8-1 0) while the radiopharmaceuticals remain near the origin (Rf = 0 0-0 3) or vice-versa This permits one to cut the strips at Rf = 0 5 (midway) and to assay the two segments (section 1 and section 2) The activity of each portion was compared with the total radioactivity of the strip Labelled hIgG has an Rf = 0

Animal biodistribution studies

Animal biodistribution was performed in normal mice Useful studies can be performed in normal animals since these will identify the effect of a normal *in vivo* environment on the labelled antibody Sufficient animals groups at sufficient time points are necessary in order to overcome intrinsic biological variations Thus 100 µl of the solution containing the ^{99m}Tc hIgG (10 30 µg approximately 50 µCi) were injected IP into normal female mice At time intervals of 30 minutes, 1-2-3-4 and 24 hrs postinjection, groups of five mice were killed and samples of blood (100 µl), the entire liver, spleen, heart, stomach with contents, femur, both kidneys and a sample of thigh muscle free of fat were dissected and counted in a well counter against a standard of the injectate The results were expressed as mean percentage of injected dose per organ

Whereas, quality assurance by animal biodistribution is valuable for conventional radiopharmaceuticals, it is not clear yet that animal biodistribution does not play a role in the quality assurance of radiolabelled antibody preparations Accumulation of the label in certain normal organ may provide information on the radiochemical purity of the injectate or the stability *in vivo* of the label while accumulation of the label in tumor will indicate immunoreactivity Biodistribution studies are most valuable when a comparison is being made between one or more labelled species, for example between an antibody labelled with different methods or between two antibody species labelled identically

Sterility and apyrogenicity

It is self evident that any preparation of a radiopharmaceutical intended for human use should be both sterile and free of pyrogens We use the US Pharmacopoeia directions for the determination of sterility which involves both thioglycolate and soybeam digest media We perform pyrogen assays using the Limulus lysate test

RESULTS

Radiolabelling

The labelling efficiency and stability of the labelled reduced hIgG as determined by instant paper chromatography up to 10 minutes and at 24hrs after labelling were greater than 99% Radiochemical purity remained essentialy unchanged for the 24 hrs after preparation in 10 radiolabelling

Animal biodistribution studies

Dissection studies in normal mice injected with ^{99m}Tc-hIgG show that biodistribution data did not reveal a specific high accumulation in any organ (Table 1)

TABLE 1

Biodistribution of ^{99m}Tc-hIgG at 30 min , 1-2-3-4 and 24 hr IP post injection in female normal mice

(Five animals per experimental group, all values reported ± sd)

ORGAN	30	1 hr	2 hr	3 hr	4 hr	24 hr
Heart	0 1446±0 0741	0 1578±0 0894	0 2768±0 1264	0 3327±0 1725	0 5413±0 0426	0 2360±0 0466
Lung	1 0702±0 7537	0 8182±0 8265	1 3828±0 8622	1 5690±0 5443	3 3570±0 8755	1 3166±0 4769
Liver	4 5130±0 9445	4 9656±4 0834	4 3348±2 0920	6 2052±2 3827	6 7428±0 3327	3.8178±0.4669
Spleen	1 6452±0 6016	2 7762±2 0312	4 3880±1 0937	4 2536±3 1887	7 0265±0 4847	3 5690±0 3784
Kidneys	0 3454±0 1034	0 4024±0 4309	0 2866±0 1868	0 3502±0 1122	0 3370±0 1844	0.4510±0 6018
Stomach	0 9181±0 5806	1 4236±1 7139	0 8316±0 4848	1 0527±0 3401	1 2670±0 3590	0 4560±0 0788
Femur	0 2142±0 1335	0 1904±0 0474	0 2234±0 0667	0 1715±0 1081	0 1920±0 0558	0.0792±0 0089
Muscle	0 1164±0 0452	0 1364±0 1169	0 1448±0 0529	0 1297±0 0714	0 1985±0 0740	0.0676±0 0127
Blood	0 4124±0 2318	0 3968±0 3049	0.8334±0 3823	1 3213±0 6981	1 5650±0 2626	0.6810±0 1351

DISCUSSION

These studies were performed using phosphate buffered saline at pH 7 4, 7 0 and 6 6 The highest labelling efficiency was obtained when PBS buffer was used at pH 7 4 and 7 0 Our experiment was done with PBS buffer pH 7 0

The conclusions from these studies are the following

1 This method affords a possible route to simple technetium labelling of antibodies and other proteins
2 hIgG can be reacted with stannous ions to produce a chemical species of the protein capable of forming a very strong bond with technetium
3 The reduced hIgG/MDP mixture can be stored frozen or lyophilized and used for subsequent radiolabelling with ^{99m}Tc, i e an "instant labelling kit"
4 The ^{99m}Tc-hIgG can be used as a radiopharmaceutical scintigraphy

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