DEVELOPMENT OF INSTANT KITS FOR ^{99M}Tc-LABELLING OF ANTI-CEA MONOCLONAL ANTIBODY AND HUMAN IMMUNOGLOBULINS FOR SCINTIGRAPHY.

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ABSTRACT

Simplicity and rapidity are highly desirable features in the development of ^{99m}Tc-labelled radiopharmaceuticals. On the basis of 2-mercaptoethanol reduction, three instant kits were formulated for preparation of ^{99m}Tc-labelled anti-CEA monoclonal antibody (IOR-CEA) used in the detection of colorectal cancers and ^{99m}Tc-labelled immunoglobulins (Sandoglobulin and Venoglobulin) for imaging of infection / inflammation in musculoskeletal system. The kits were sterile and demonstrated to be pyrogen free and had a shelf life of at least 1 year. Efficient labelling (>95% efficiency) could be achieved in 15 min at room temperature. All radiolabelled products exhibited 4-hour biodistribution patterns similar to those reported in literatures, i.e high blood background owing to long half life of IgG in plasma and high renal uptake because of in vivo cysteine transchelation. IOR-CEA was more resistant to cysteine challenge than Sandoglobulin and Venoglobulin. This facilitated the abdominal imaging using IOR-CEA. While the high renal activity in associating with Sandoglobulin and Venoglobulin did not interfere with the investigation of extremities. The costs for kit preparation were 40 times cheaper than those from commercial sources.

The instant kits developed in this study permitted the expensive radioimmunoscintigraphy within the reach of developing countries like Thailand where per capita incomes are far below the global standard.

INTRODUCTION

Development of simple methods for in-house preparation of radiopharmaceu-ticals is desirable for practical uses of the diagnostic agents in clinics. ^{99m}Tc-labelled monoclonal antibody (Mab) and human immunoglobulin G (HIG) have presently emerged as a class of site-specific radiopharmaceuticals in nuclear medicine diagnosis of malignant diseases[1] and detection of infection / inflammation process.[2] The reagents are safe for patient injection since they cause no

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biolo-gical hazards like the use of radiolabeled blood cells. Among a large number of methods used for labeling the antibody molecules with ^{99m}Tc,[3] reduction-mediated direct labelling method appears to be technically simple and produces a highly stable product with preserved immunological function.[4] The most attractive feature of the method is the high labelling efficiency which oviates the need for post-labelling purification. Several instant kits are now available commercially but at high prices.

We had previously conducted studies to understand the chemistry of ^{99m}Tc labelling for a number of antitumor Mabs and polyclonal HIG by reduction-mediated direct labelling method.[5] In this study, instant kits were formulated for labelling an anti-CEA monoclonal antibody (IOR-CEA) and two HIGs (Sandoglobulin and Venoglobulin) from different sources. The kits were evaluated for their radiochemical purity, stability towards cysteine challenge, apyrogenicity, shelf life, and in vivo biodistribution.

MATERIAL AND METHOD

ANTIBODIES AND IMMUNOGLOBULINS

IOR-CEA (IgG_1), M170 (IgG_1) and EMD (IgG_{2a}) are murine monoclonal antibodies which react to different types of tumors. IOR-CEA, an anti-CEA antibody, donated by the Center of Molecular Immunology (Havana, Cuba) was chosen for kit development because of its good radioimmunochemistry.[6] M170 reacting to cytokeratin on most human adenocarcinomas was a gift from Biomira Inc. (Edmonton, Alberta, Canada). EMD, an anti-epidermal growth factor (EGF) receptor was kindly donated by Dr Baum (Goethe University Hospital, Germany). Sandoglobulin (Sandoz Pharma Ltd, Basle, Switzerland) and Venoglobulin-I (Alpha Therapeutic Corporation, Los Angeles, U.S.A.) are polyclonal immunoglobulins having the same IgG subclass distributions as those IgG's in normal plasma.

ANTIBODY REDUCTION AND RADIOLABELLING

Under aseptic condition, IOR-CEA, Sandoglobulin and Venoglobulin at concentrations between 7-10 mg/ml in neutral phosphate buffered saline (PBS) were reduced by 2-mercaptoethanol (2-ME) with molar excess of 500:1 and 1000:1 at room temperature for 30 min. The reduced antibodies were purified on a sterile G-50 Sephadex column (Pharmacia, Uppsala, Sweden). Five hundred μg of antibody sample was labelled with 370 or 740 MBq of ^{99m}Tc. The medronate kit containing 1 mg of methylene diphosphonate (MDP) and 0.068 mg of SnF_2 in 1 ml solution was used to form ^{99m}Tc-MDP chelate for antibody labelling.

Radiochemical species were determined by instant thin layer chromatography (ITLC).[7,8] One μl of the labelled antibody solution was spotted onto a 1 x 8 cm ITLC strip. By using different chromatographic materials and solvents (Table 1), percentage of radioactivity was determined for different forms of radiochemical species, i.e. free pertechnetate ($^{99m}TcO^-_4$), ^{99m}Tc-MDP, hydrolysed reduced technetium (HR-^{99m}Tc) and ^{99m}Tc-labelled IgG. Radiolabelling efficiency was defined as percentage of ^{99m}Tc radioactivity tagged to IgG molecules.

Table 1. Instant thin layer chromatographic system for measuring different radiochemical species.[7,8]

Media	Solvent	Impurity	Location
Whatman 31 ET paper	Acetone	$^{99m}TcO_4^-$	Solvent front
ITLC-SG	Normal saline	$^{99m}TcO_4^-$ + ^{99m}Tc-MDP	Solvent front
HSA-impregnated ITLC-SG	EtOH:NH_4OH:H_2O (2 : 1 : 5)	HR-^{99m}Tc	Origin

HR-^{99m}Tc = hydrolysed reduced technetium

CYSTEINE CHALLENGE ASSAY

Stability of the ^{99m}Tc-labelled antibody was challenged with cysteine at concentrations 0, 0.0138, 0.138 and 1.38 mM for 1 hour at 37°C. Chromatographic analysis was performed on a 1 x 8 cm strip of Whatman No. 1 paper using 0.1 M PBS, pH 7, as an eluant. After development and drying, the strip was cut into two halves for gamma counting. In this system, labelled antibody remains at or near the origin, labelled cysteine migrates to the solvent front.[9]

ANIMAL BIODISTRIBUTION STUDIES

One hundred µl of ^{99m}Tc-labelled antibody solution containing 30 µg of IgG with activity around 1850 KBq was injected intraperitoneally into 5 normal balb/c female mice weighing 19.5 ± 2.3 g. Four hours after injection, the animals were sacrificed by spinal dislocation and samples of blood, the entire liver, spleen, heart, stomach with contents, femur, both kidneys and a sample of thigh muscle free of fat were removed. All tissues were rinsed in cold saline immediately after removal, patted dry and weighed before counting against a standard of the injectate.

LIMULUS AMEBOCYTE LYSATE (LAL) TEST.

The amount of bacterial endotoxin present in the frozen kit was determined by LAL test (Biowhittaker, Walkersville, MD, U.S.A) which utilizes a co-lyophilized mixture of LAL and a synthetic color producing substrate to detect endotoxin by measuring the colored product, p-nitroaniline at 405 nm. The test was performed with a 96-well microplate. On the basis of endotoxin standards, endotoxin unit (EU) in the antibody sample could be calculated. By U.S.P. standard,[10] no patient will receive more than 175 EU of endotoxin in a worst-case scenario.

SHELF LIFE OF THE INSTANT KITS.

The three instant kits, i.e. IOR-CEA, Sandoglobulin and Venoglobulin, were stored at -20° C and thawed after 1, 3, 6, 9, 12 months of storage to investigate for their physical appearance and labeling efficiency.

RESULTS

ANTIBODY REDUCTION AND CYSTEINE CHALLENGE

Optimal concentration of 2-mercaptoethanol (2-ME) was firstly established for each antibody. To achieve clinically useful specific activities, i.e. 740-1480 MBq/mg, 500:1 molar excess of 2-ME was required for generating ^{99m}Tc reactive sites in Sandoglobulin while IOR-CEA and Venoglobulin required twice as much of 2-ME (Table 2). ^{99m}Tc- labelled antibodies mediated by 2-ME reduction yielded the products which were stable for several hours after preparation. Less than 10% of ^{99m}Tc were lost when the labelled antibodies were incubated for 24 hours at 37° C either in PBS or serum proteins (Data are not present). Nevertheless, ^{99m}Tc-labelled antibodies were reported to be susceptible to in vivo trans-chelation via cysteine and excreted by kidney.[9,11] To understand the nature of ^{99m}Tc binding site induced by 2-ME, 4 antibodies of different reduction sensitivity (i.e. Sandoglobulin> Veno-globulin > IOR-CEA > EMD)[5] were challenged to a range of cysteine concentrations varying from 0, 0.0138 to 1.38 mM. On an overall basis, ^{99m}Tc-labelled Mabs were more stable to cysteine (0.138 mM upward) than polyclonal HIGS ($p<<0.001$) (Table 3). Order of stability could be ranked as follows : IOR-CEA > EMD > Sandoglobulin > Venoglobulin.

Table 2. 2-Mercaptoethanol(2-ME) - reduction of the antibody molecules for ^{99m}Tc-labelling.

Molar ratio 2ME : IgG	^{99m}Tc added (MBq / mg)	Labelling efficiency (%) IOR-CEA	Sandoglobulin	Venoglobulin
500 : 1	740	48.17 ± 6.40	99.39 ± 0.16	98.56 ± 0.23
	1480	NA	99.13 ± 0.14	90.45 ± 7.88
1000 : 1	740	99.66 ± 0.08	99.28 ± 0.22	99.00 ± 0.10
	1480	99.05 ± 0.38	98.85 ± 0.34	98.38 ± 0.54

NA = not available.

Table 3. In vitro stability to cysteine challenge of ^{99m}Tc-labelled monoclonal antibodies and human immunoglobulins. The data, mean ± SE(N = 5), present the percentage of radioactivity transchelated to cysteine molecules.

Cysteine : IgG	Concentration of Cysteine (mM)	Monoclonal Antibodies IOR-CEA (IgG_1)	EMD (IgG_{2a})	Polyclonal Antibodies Sandoglobulin	Venoglobulin
0	0	5.57 ± 0.07	4.20 ± 0.50	3.88 ± 0.20	3.03 ± 0.19
5 : 1	0.0138	4.92 ± 0.15	6.91 ± 1.15	4.83 ± 0.35	6.98 ± 0.19
50 : 1	0.138	14.21 ± 0.93	14.41 ± 0.36	26.65 ± 1.84*	40.79 ± 3.56*
500 : 1	1.38	34.40 ± 1.04*	50.04 ± 1.42*	55.50 ± 0.92*	74.48 ± 2.21*

* Significant difference with $p << 0.001$

RADIOCHEMICAL PURITY AND IN VIVO BIODISTRIBUTION

The instant kits developed in this study yielded ^{99m}Tc-labelled antibodies of high radiochemical purity (>95%). Less than 5% of the activity appeared as impurities in forms of $^{99m}TcO^-_4$, ^{99m}Tc-MDP and HR.-^{99m}Tc (Table 4). Therefore, no remarkable uptake by thyroid, stomach or reticuloendothelial system could be observed upon injection.

In animal study, high blood background and high kidney uptake were common findings for all labelled antibodies investigated here (Table 5). ^{99m}Tc-labelled IOR-CEA which was more resistant to cysteine transchelation was found to have lower kidney uptake than Sandoglobulin and Venoglobulin ($p<<0.001$). Similar biodistributions were observed between two Mabs (IOR-CEA, M170) which belong to the same IgG_1 subclass, although their 2-ME reduction sensitivities were different.[5] Interestingly, Venoglobulin which was more vulnerable to in vitro cysteine transchelation displayed lower kidney activity than Sandoglobulin ($p<0.05$). Significantly higher blood background and heart activity were also noted ($p<<0.001$). The presence of ^{99m}Tc-labelled human serum albumin (HSA) was postulated to be the cause. In compounding Venoglobulin, 0.2 g of HSA was added per g of IgG as a carrier.[13] In our laboratory, effective labelling of ^{99m}Tc to HSA could also be achieved by 2-ME reduction at 500:1 molar excess. Since ^{99m}Tc-labelled HSA is a blood pool agent for dynamic cardiac function studies,[14] this might be accounted for the remarkable blood and heart activities for Venoglobulin sample. Because HSA is a normal blood component, excretion of ^{99m}Tc-labelled HSA by kidney can not be observed early.[14] On the contrary, kidney has been suggested as a site for catabolism of ^{99m}Tc-labelled IgG by direct approach.[11] Lower kidney uptake of ^{99m}Tc-labelled Venoglobulin in comparing to Sandoglobulin ($p<0.05$) indicated a more favorable in vivo stability for IgG in Venoglobulin. Instability of ^{99m}Tc-HSA towards in vitro cysteine challenge might be responsible for the loss of activity from the Venoglobulin sample.

Table 4. Distribution of different radiochemical species for the three instant kits.

Readiochemical Species	Instant Kit			Site of uptake
	IOR-CEA[a]	Sandoglobulin[b]	Venoglobulin[b]	
$^{99m}TcO_4$	0.84 ± 0.31	0.53 ± 0.12	0.09 ± 0.03	Blood, thyroid, stomach salivary gland, etc[12]
^{99m}Tc-MDP	0.69 ± 0.13	1.10 ± 0.10	0.34 ± 0.01	Bone[12]
HR-^{99m}Tc	0.55 ± 0.14	2.07 ± 0.31	1.10 ± 0.18	Reticuloendothelial system (liver, bone marrow, spleen)[12]
^{99m}Tc-IgG	97.92 ± 0.39	96.30 ± 0.66	98.47 ± 0.24	Colon cancer[(a)] Infection / inflammation[(b)]

Table 5. The four-hour biodistributions of ^{99m}Tc-labelled monoclonal antibodies and human immunoglobulins in normal balb/c mice. The data, mean ± SE (N = 5) are expressed as percentage of injected dose per gram of tissue.

Organ	Murine Monoclonal Antibody		Polyclonal Human Immunoglobulin G	
	IOR-CEA (IgG_1)	M170 (IgG_1)	Sandoglobulin	Venoglobulin
Blood	16.60 ± 1.53	14.25 ± 0.78	13.32 ± 1.86	19.61 ± 1.21
Liver	5.02 ± 0.51	5.86 ± 0.38	3.82 ± 0.47	5.12 ± 0.36
Spleen	4.95 ± 0.34	5.41 ± 0.42	4.50 ± 0.43	5.63 ± 0.18
Heart	4.78 ± 0.42	3.92 ± 0.23	4.45 ± 0.28	7.79 ± 0.34
Stomach	2.46 ± 0.27	3.13 ± 0.17	3.09 ± 0.45	2.38 ± 0.27
Kidney	8.47 ± 0.57	8.88 ± 0.52	23.24 ± 2.27	16.00 ± 0.83
Femur	1.79 ± 0.17	2.04 ± 0.33	1.47 ± 0.20	2.10 ± 0.56
Muscle	1.68 ± 0.18	1.12 ± 0.16	1.04 ± 0.12	1.10 ± 0.10

APYROGENICITY AND SHELF LIFE

For commercially avialable instant kits, the modified antibody is supplied in lyophilized form which is quite suitable for large scale manufacturing. In kit preparation for in house use, it is more convenient to prepare the frozen kit. Procedure for kit preparation by aseptic technique is described in Table 6. Apyrogenicity of the instant kit was assessed by bacterial endotoxin level using chromogenic LAL assay. For each antibody, three batches of instant kits were assayed for endotoxin level. Endotoxin units in each kit were well below the limit recommended by U.S. Pharmacopia[10] (Table 7). All the frozen kits stored at -20° C were highly stable. Neither changes in labelling efficiency nor in physical appearance of the antibody solution could be observed over a peroid of one year (Table 8).

Table 6. Standard procedure for preparing instant kits for labelling the anti-CEA monoclonal antibody and human immunoglobulins with ^{99m}Tc.

I. Antibody reduction under aseptic condition

1 Adjust or concentrate in case of diluted solution, the 10mg antibody to a concentration of 5-10 mg/ml.

2 Add 2-ME to provide a 1000 : 1 of 2 ME : IgG molar ratio and allow the reaction to proceed for 30 min at room temperature with intermittant shaking.

3 Purify the reduced antibody with a prechilled Sephadex-G50 column (2 x 8 cm) using N_2-purged PBS as mobile phase.

4 Assay the antibody peak by spectrophotometer at 280 nm, based on $E^{1\%}_{1cm} = 14.3$.

5 Adjust the concentration of the reduced antibody to > 0.5 mg/ml[5] and sterilize the antibody solution by 0.22 μm membrane filter (hydrophilic Durapore Membrane, Millipore).

6 Fractionate the antibody solution into 2 ml aliquots.

7 Purge the antibody sample for 2 min with sterile N_2 gas before freezing at -20° C.

II. Radiolabelling by sterile technique

8 Thaw the frozen sample.

9 Add 15 μl of MDP-SnF_2 to the antibody sample and mix gently.[5]

10 Add 740 MBq ^{99m}Tc-pertecnetate to antibody / MDP mixture and allow the reaction to undergo at room temperature for 15 min.

11 Assess labelling efficiency (should be >95%) by ITLC.
(The labelled antibody was highly stable for many hours after labelling.)

Table 7. Apyrogenicity test by chromogenic limulus amebocyte lysate (LAL) assay.

Instant kit	Endotoxin (EU/mg IgG)
IOR-CEA	less than 59.9 ± 42.4
Sandoglobulin / Venoglobulin	less than 9.7 ± 0.2

Endotoxin limit : USP < 175 EU / mg.[10]

Table 8. Long-term stability of the three instant kits assessed by ^{99m}Tc-labelling efficiency.

Month of Storage	Labelling Efficiency (%)		
	IOR-CEA	Sandoglobulin	Venoglobulin
1	98.36 ± 0.08	94.05 ± 1.09	97.81 ± 0.81
3	98.35 ± 0.08	98.42 ± 0.81	97.82 ± 0.02
6	97.06 ± 0.13	97.37 ± 0.16	99.16 ± 0.39
9	96.25 ± 0.62	99.15 ± 0.74	99.29 ± 0.18
12	98.84 ± 0.12	97.01 ± 0.19	98.72 ± 1.74

All the instant kits stored over a period of one year appeared as clear solutions after thawing.

DISCUSSION

Considerations in development of instant kit for in-house preparation of radiopharmaceutical using short-lived radionuclide like ^{99m}Tc based upon the following factors : (1) yield of the desirable radiochemical species; (2) simplicity, reproducibility and cost of the instant kit; (3) biodistribution behaviour of the radiopharmaceuticals; (4) shelf-life and apyrogenicity. For the three labelling kits formulated in this laboratory, effective labelling was highly reproducible. No changes in labelling efficiency observed over 12-month storage. The procedures for kit preparations were simple and rapid. It took one day to reduce the antibody to make a batch of instant kits for 3-4 month use. Radiolabelling required only 15 min of reaction at room temperature. Post labelling purification was not required since labelling efficiency exceeded 95%. The kits were pyrogen free and safe for patients injection. IOR-CEA kit and Venoglobulin kits are now used by our institute for detection of colorectal cancers[15] and localization of infectious foci in musculosketal system.[16] Both kits had shelf life of not less than 1 year. In comparing to the price of a commercial kit for infection / inflammation imaging, the cost for preparation of Venoglobulin kit as well as Sandoglobulin was 40 times cheaper.

IOR-CEA, Sandoglobulin and Venoglobulin displayed typical biodistributions in balb/c mice, i.e. high blood background and high kidney uptake, which were the common findings for ^{99m}Tc-labelled antibody by direct approach.[4, 17-20] However, high liver uptake was reported in animals bearing tumor,[4,19] or with abcess[17, 18, 20] as well as in patients.[15, 16] Beaty et al[21] demonstrated that much of the liver activity was related to metabolism of the immune complex sequestrated from the blood stream. Absence of antigen-antibody complex in normal mouse model could be accounted for low liver uptake for the kits developed in this study. IgG has long resident time in blood circulation (human IgG = 23 day,[22] mouse IgG = 4 day.[22]) This could explain the observation of high blood background at 4 hours after injection. In our institute, high blood pool activity was neither a problem for imaging of infection / inflammation process in the musculoskeletal system[16] nor prevented the sucessful localization of colorectal cancers owing to their

high target-to-background ratios.[15]

Our labelling kits, like those reported in literatures,[4, 23] were quite stable towards in vivo oxidation of the reduced ^{99m}Tc to free pertechnetate or transchelation to serum proteins except for sul -fhydryl containing molecules such as cysteine[9, 11] which is present at high concentration in plasma (10 μM[24]) and tissue (10-100μM.[25]) Antibody catabolism in kidney or transchelation of the radiolable was identified as the cause of high renal uptake.[11] In vitro cysteine challenge assay did not predict in vivo stability of Venoglobulin kit due to the presence of ^{99m}Tc-labelled HSA. Nevertheless, lower kidney uptake of Venoglobulin in comparing to Sandoglobulin suggested that ^{99m}Tc-labelled Venoglobulin might be a better imaging agent.

Localization of the human IgG is postulated to be dependent of increased vascular permeability, bacterial recognition, Fc mediated binding to both granulocytes and microorganisms at the infectious site.[26] Some IgG such as Sandoglobulin may receive enzymatic treatment during manufacturing and leads to damaged Fc regions.[27] Dormehl et al[18] observed the greater uptake by the inflammatory foci of the Gammagard IgG with intact Fc than Sandoglobulin IgG of which the activity of Fc portion was impaired during production. For Venoglobulin, the production process involves no enzymatic modification. The molecule is reported to retain its full biological functions in opsonizing, complement binding and Fc-receptor binding of polymorphonuclear cells.[13] For these reasons, Venoglobulin kit was chosen for clinical uses.

ACKNOWLEDGEMENTS

This study was partly supported by World Health Organization (WHO) and the International Atomic Energy Agency (IAEA).

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