9 December 1997

First Research Co-ordination Meeting of the Co-ordinated Research Programme on :

DEVELOPMENT OF KITS FOR RADIOIMMUNOMETRIC ASSAYS OF TUMOUR MARKERS

IAEA RESEARCH CONTRACT NO. IRA / 9820

DEVELOPMENT OF IRMA REAGENT AND METHODOLOGY FOR PSA.

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Proposed Procedures .

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1 - ACTIVITIES AND EXPERIMENTS IN RADIOISOTOPE DEPARTMENT

Radioisotope Department of NRC consists of 3 main groups :

1 - PRODUCTION
2 - QUALITY CONTROL
3 - INDUSTRIAL APPLICATIONS

Production Group is divided into following sections and laboratories :

- Industrial radioisotope lab , produces Co-60 and Ir-192 for gauging methods and gamma Radiography.
- radiopharmaceutical lab , provides all necessary radiopharmaceuticals and radioisotopes for nuclear medicine centers such as Tc-99m Generators , Sodium Iodide (I-131) , Sodium Phosphate (P-32), Chromic Phosphate Colloid (P-32).
- Invivo kit preparation lab , produces all types of Tc-99m Kits including MIBI , ECD , EC , etc.

We are also going to set up two new labs , one for Monoclonal Antibodies Labeling and the other will be specialized for Monoclonal Antibody Production , using in Radiopharmaceuticals and RIA & IRMA Kits .
Finally , RIA lab that now is involved for implementation of the project , has started it's experiments by labeling the thyroid hormones and TSH monoclonal antibody.
Our scientific staff in this section have good and enough knowledge and are well trained during last 2 - 3 years in the field of I-125_labelling , preparation and quality control of polyclonal antibodies , coating techniques of antibodies on beads and other relevant experiences .
We are largely indebted to the IAEA for supporting related projects by arranging experts mission ,training our staff and supplying some necessary equipments and materials to implement these projects .

2 - BACKGROUNDS

The PSA test is a solid phase two-site immunoassay . Rabbit anti PSA is coated or bound on surface of solid phase and monoclonal anti PSA labeled with I-125 . The PSA molecules present in the standard solution or serum are " Sandwiched " between the two antibodies . After formation of coated antibody-antigen-labeled antibody complex , the unbound labeled antibody will removed by washing . The complex is measured by gamma counter. The concentration of analyte is proportional to the counts of test sample .
In order to develop kits for IRMA PSA , it should be prepared three essential reagents : Antibody coated solid phase, labeled antibody, standards and finally optimizing them to obtain an standard curve fit to measure specimen PSA in desired range of concentration .
The type of solid phase and procedure(s) to coat or bind to antibody , is still main debatable subject in development and setting up RIA / IRMA kits .

In our experiments , polystyrene beads , because of their easy to coat with antibody as well as easy to use , can be considered as a desired solid phase .
Most antibodies are passively adsorbed to a plastic surface (e.g. Polystyrene , Propylene , and Polyvinyl chloride) from a diluted buffer . The antibody coated plastic surface , then acts as solid phase reagent . Poor efficiency and time required to reach equilibrium and also lack of reproducibility especially batch-to-batch variation between materials , are disadvantages in this simple coating procedure .
Improvements can be made by coating second antibody on surface of beads , and reaction between second and primary antibodies .
There is also possible to enhance more coating efficiency of beads by using Staphylococcus - aureus-Protein A.
Protein A is a major component of staphylococcus aureus cell wall which has an affinity for FC segment of immunoglobulin G (IgG) of some species , including human ; rabbit ; and mice . This property of Staphylococcal Protein A has made it a very useful tool in the purification of classes and subclasses of Igs' .

3 - WORKPLANE

Based on this background , the following working plan for the first year is formulated :

3 - 1 - PREPARATION OF COATED BEADS

TEST 1 : Direct coating

1 - Add enough volume of 1 , 10 , 100 μ g / ml IgG against PSA solution in carbonate buffer Ph : 9.6 ,degassed and leave at 4° C , 25° C , 37° C overnight.
2 - Aspirate IgG solution from beads , add wash buffer (PBS + 0.01% Triton + 0.01% Sodium azide) , aspirate again .
3 - Add 1% BSA (Bovin Serum Albumin) solution of carbonate buffer to block remaining binding sites , leave it at 25° C overnight .
4 - Wash with wash buffer by pouring and aspiration for three times .
5 - Add Glazing solution , aspirate , dry using freeze dryer , store at 4° C with desiccant .

PBS : Phosphate buffer 0.025 M , Ph = 7.4 + 0.15 M NaCl
Glazing Solution : 2% Mannitol + 1% Lactose

TEST 2 : Coating with affinity purified second antibody

At first , add second antibody to beads , then PSA antibody , and finally compare with TEST 1 results.

1 - Add sheep anti-rabbit serum (diluted 1 : 20 in carbonate buffer PH = 9.6) and after degassing , leave at 25° C for 3 overnight .
2 - Aspirate , add wash buffer and aspirate again .
3 - Repeat steps 3 , 4 as described in test 1 .
4 - Add PSA polyclonal antibody at different dilutions (e.g. as noted on no. 1 at test 1) in carbonate buffer Ph = 9.6 , degassed and leave for overnight .
5 - Wash three times , glaze , aspirate , dry using freeze dryer , store at 4° C with desiccant .

TEST 3 : Coating with Staphylococcus Aureus Protein A

At first , coat beads using staphylococcus aureus protein A , then repeat procedure listed in test 2 .

1 - Add protein A to beads at different dilutions (e.g. 1 , 3 , 5 , 7 , 10 μ g / ml) in coating buffer. Store at 25° C . Aspirate , Add blocking solution (1% BSA in coating buffer) , leave for 2 - 3 hours , remove blocking solution . Wash three times with wash buffer .
2 - Add sheep anti-rabbit serum at different dilutions (e.g. 1:20 , 1:40 , 1:80 in PBS + 0.1% BSA) leave in overnight at 25° C . Wash three times with wash buffer .
3 - Add PSA antibody at different dilutions (e.g. 1:2000 , 1:4000 , 1:6000 , 1:8000 in PBS + 0.1% BSA or carbonate buffer) , leave for overnight at 25° C , wash three times with phosphate buffer.
Finally add glazing solution , freeze dry , store at 4° C with desiccant .

3 - 2 - PREPARATION OF I-125_LABELLED PSA MONOCLONAL ANTIBODY

Chloramin-T technique is method of labeling hormones and peptides used in RIA / IRMA kits . Purification of radioiodinated PSA monoclonal antibody is performed by chromatography on sephadex gels . A 30 × 0.9 cm column of G-25 sephadex is suitable for this purification . Apparently using 1 mCi I-125 / 50 u g PSA monoclonal antibody / 20 u g chloramin-T and 20 u g sodium metabisulphite to stop the reaction can be considered as basic procedure . The main following parameters , should be thoroughly investigated , in order to obtain the maximum labeling efficiency as well as lowest damage in immunoreactivity :
Reaction Ph (7 - 9) ;
reaction incubation time (10 - 60 sec) ;
specific activity and etc.
Due to relatively large molecules of PSA monoclonal antibody and probability loss of immunoreactivity , it is recommended to consider alternative labeling methods , first evaluate N-bromosuccinimide method and if not satisfied , use solid phase lactoproxidase . For purification , using 60 × 0.9 cm column of sephadex superfine G-100 can be considered if the former smaller column had not shown enough resolution for separating labeled PSA monoclonal antibody .
Buffer for the reaction is 0.5 M phosphate buffer and for the elution is 0.05 M phosphate buffer contains 0.1% BSA and 0.02% Sodium Azide .

3 - 3 - PREPARATION OF STANDARDS

PSA standards are prepared by adding purified PSA in 0.1 mol / L Phosphate Buffer Saline (PBS, Ph = 7.4) to freshly drawn female serum to a final concentration of 200 u g / L , let the PSA equilibrate with endogenous protease inhibitors for at least 48 h. at 4° C overnight.
Add the prepared serum-complexed PSA stock to fresh female serum to final concentration typically 100 , 50 , 25 , 10 , 2 ng / ml .

3 - 4 - ASSAY PROCEDURE

The assay is carried out at ambient temperature at two stages .
At first PSA is eluted from specimen and reacted with anti-PSA coated beads , then by adding I-125_PSA monoclonal antibody " sandwich " phenomenon occurs . After removing unbound tracer , beads are counted by gamma counter .

Typically the following assay protocol can be selected to conduct :

1 - Use 20 - 50 µ l standards / sample , 200 - 300 µ l 0.05 M phosphate buffer Ph = 7.4 in assay tubes. After adding one coated bead , incubate for 2 hours at room temperature .
2 - Aspirate , washing by wash buffer , add I-125_tracer solution and incubate at room temperature , aspirate , washing by wash buffer twice , count for 100 seconds .

The optimum incubation time and temperature as well as effect of shaking during incubation , should be checked .
IRMA standard curve is set up according to above assay protocol (B/B0 % against standard concentration).Obtaining Q.C. parameters e.g. nonspecific binding , sensitivity and reproducibility by inter and intra assay variations and compare with commercial available or established methods.

4 - SCIENTIFIC NOTES

" PSA " is a neutral serine protease actively split the seminal vesicle proteins seminogelin I and II . Thus liquefying the seminal coagulum . PSA like other serine proteases is mostly complexed with serum proteins . Alpha-2-Macroglobulin (AMG) and Alpha-1-Antichymotrypsin (ACT) which are two of the major protein protease inhibitors in blood bind strongly with PSA molecules . This binding causes differences in values obtained with anti-PSA immunoassay and not get the real value of individual PSA concentration .
Free and ACT-bound forms of PSA are detectable by current PSA immunoassay but the PSA bound to AMG is so encapsulated by this protein that it's epitopes can not react with antibody . According to the present scientific reports , that patients with prostate cancer have higher ratio of PSA-ACT to free PSA than patients with Benign Prostate Hyperplasia (BPH) .
For diagnosis of prostate cancer exclusively , it may be desirable to develop a PSA Immunoradiometric Assay that detect the free form of PSA and the complexed form of PSA separately . In order to achieve this aim , it is suggested to use monoclonal antibody to PSA as the capture solid phase and monoclonal antibody to 1-ACT labeled with I-125 . Performing coating and assay procedures are the same as described earlier. By using the referred antibodies , it is expected to assay complexed PSA . It is also possible to assay free PSA by utilizing a monoclonal antibody to an epitope of PSA that is hidden by 1-ACT .

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