Chapter 1
Introduction

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1.1.Tumor markers:

Tumor markers defined as biochemical substances that are produced by tumors or produced by the host in response to a tumor that can be used to differentiate a tumor from normal tissue. They can be detected in the serum or other body secretions. They can be measured qualitatively or quantitatively by chemical, immunological, or molecular biological methods to identify the presence of a cancer[1,2,3].

1.1.1. Historical back ground of tumor markers:

The first tumor marker reported was the Bence-Jones protein[4]. Since its discovery in 1847 by precipitation of a protein in acidified boiled urine , the measurement of Bence-Jones protein has been a diagnostic test for multiple myeloma (a tumor of plasma cells) . More than 100 years after its discovery, the Nobel prize-winning studies of Portor and Edelman and Poulik[5] identified the Bence –Jones protein as the monoclonal light chain of immunoglobulin secreted by tumor plasma cells. Monoclonal paraproteins appear as sharp bands in the globulin area electrophoretic patterns of serum. Diagnosis of multiple myeloma is an elevated levels of monoclonal immunglobulin in the serum[6].

The first period of tumor marker history was the era of the Bence-Jones protein. The second era, from 1928 to 1963, included the discovery of hormones

enzymes, isoenzymes and proteins and their application to the diagnosis of cancer and the beginning of the chromosomal analysis of tumors. Occasionally, such markers were useful in the diagnosis of individual tumors, but the general application of tumor markers were for monitoring cancer. Patients did not start untill the third era with the discovery of AFP in 1963 and carcinoembryonic antigen (CEA) in 1965[7]. The production of such markers during fetal development, as well as in tumors, led to the use of the term onco developmental markers. The fourth era started in 1975 with the development monoclonal antibodies and their subsequent use to detect oncofetal antigens and antigens derived from tumor cell lines. Examples are carbohydrate antigens, such as CA-125, CA15-3 , and CA 27.29.

Finally advances in molecular genetics in using molecular probes and monoclonal antibodies to detect chromasome or protein alteration, including the study of oncogenes, suppressor genes, and genes involved in DNA repair , have led to rapid understanding and use of tumor markers at the molecular level. These markers are becoming increasingly useful at the cellular level [8].

1.1.2. Classification of tumor markers:

Tumor markers can be broadly classified into:

I- Tumor specific markers e.g. B-cell tumor immunoglobuin idiotype, Virus induced antigens e.g. SV40T antigen, T-cell receptor of T-cell leukemia.

II- Tumor-associated markers:

a- Tumor with low molecular weight markers (less than 1000D) e.g. Polyamines

b- Macromolecular markers (more than 1000D):

1- Enzymes and isoenzymes: eg. Placental alkaline phosphatase.

2- Hormones, Cytokines, growth factor and soluble receptors e.g HCG, estrogen and progesterone.

3- Oncogenes and oncoproteins e.g. c-myc.

4- Oncofetal protein e.g. CEA, and AFP.

5- Glycoproteins and glycolipids eg CA-125 and CA19.9

6- Cellular markers eg. Philadelphia chromosome[3,9,10]

Hepatocellular carcinoma (HCC) is the seventh most common cancer in men and the ninth most common cancer in women, with an estimated incidence of between 250,000 and 1.2 million per year worldwide. It is a highly malignant tumor with a poor prognosis. The poor prognosis has been attributed to late diagnosis. An effective screening system to detect HCC at an early stage may result in more effective treatment. Symptoms in the early stage of HCC make screening of patients at risk for HCC impractical[11,12].

An ideal tumor marker should be characterised by 1) detection in all tumor stages 2) cancer specificity 3) reasonable rapid clearance 4) correlation with

tumor load and activity. The oncofetal antigens are protein produced during fetal life. These proteins are present in high concentration in the sera of fetuse and decrease to low levels or disappear after birth. In cancer patients, these protein reappear. The production of these proteins demonstrates that certain genes are reactivated as the result of the malignant transformation of cells [13].

The oncofetal antigen AFP was first found in the sera of mice with liver cancer by Abelev and associates[14] and later by Tatarinow[15] in human sera with hepatocellular carcinoma.

1.2. Alpha Feto Protein (AFP) as a tumor marker:

AFP is a marker for hepatocellular and germ cell carcinoma. It is an oncofetal glycoprotein with molecular mass 70 KD molecule with considerable (30%) structural homology with human albumin whereas it is similar to serum albumin in size, structure and amino acid composition but has distinct immunological properties[16]. It consists of a single polypeptide chain and it has an isoelectric point of 4.7 to 4.8. It has a serum biological half life of about 4 to 6 days and it migrates with alpha globulins on electrophoresis. It synthesized by the liver, yolk sac and gastrointestinal tract of the fetus reaching a peak serum concentration of up to 10 mg/ml at 12 weeks of gestation. This peak level gradually decreases and, one year after the birth of the newborn, the serum levels decrease to less than 25 ng/ml. Albumin becomes the major serum component in

adult serum with concentrations up to 60 mg/ml. The normal level of AFP is from 2 to 16 ng/ml and it reaches 550 ng/ml in late pregnancy.

Measurement of serum AFP is important in the diagnosis of several pathological states and is particulary useful as a screening test in the antenatal diagnosis of fetal neural tube defects, which may be indicated by high concentrations of AFP in maternal serum.

Amniotic fluid AFP estimation is being used increasingly in the early prenatal diagnosis of open neural tube defects. Pregnancies at risk because of previously affected child, can now be tested at about 16 weeks gestation by aminocentesis and, in affected cases which comprise 5 to 10 percent of these tests, the aminotic AFP may be ten times the normal levels [17].

A major use of AFP as a tumor marker has been in the diagnosis, staging, and post-therapeutic monitoring of non seminomatous germ cell tumors of the testis. Amniotic fluidAFP does not raise in closed neural tube defects, but the test appears reliable for open defects and elevated levels are widdy accepted as an indication for termination of pregnancy. Serum levels gradually decline to 13-36 μg/ml at birth and further to a mean normal adult level of 2-3 ng/ml by two years ago[17].

Maternal serum AFP levels however, progressively increase as pregnancy advances. Increased interest in the measurement of AFP by clinical laboratories related to high levels found in the amniotic fluid and maternal serum when the fetus has an open neural tube or certain other birth defects, as well as to high levels in the serum of the patients with primary liver or germ cell cancers[18].

Lau & Linkens (1976)[19] and Seppala & Ruoslahti (1979)[20] have reviewed analytical methods used to measure AFP. These methods vary in complexity, specificity and sensitivity. The performance characteristics of a particular method may be largely dependent upon the clinical application. To be useful for the detection of neural tube defects, as well as for evaluating patients undergoing therapy for certain malignancies, the method must be very precise and sensitive.

1.3. Determination of hormones and tumor markers:

Immunoassays are the most widly used analytical techniques and have been successfully applied to an extensive range of substrates, including both large and small molecules, cells, cellular components and viruses. They depend on the use of selected specific antibodies as reagents. Because antibodies can display high specificity and can react with high affinity, immunoassays are capable of measuring substances ion complex matrices without pretreatment, extraction, purification or concentration. This simplicity of application and the concomitant high through put are essential aspect of immunoassays [21].

1.3.1. Non –isotopic immunoassay:

Considerable effort has been directed towards the development of alternative technologies that do not rely on radiolabels. A number of non-isotopic immunoassays for hundreds of analyses are currently available and their use continues to increase. Many of these procedures have been developed for use on fully automated immunoassay systems and some are compatible with existing chemistry analyzers. Assay systems that require separation of bound and free phases after incubation are referred to as a heterogeneous and are more sensitive than those that do not require separation (homogeneous)[22]. Specific examples of several of these assays are as follows.

1.3.1.1. Enzyme Immunoassay (EIA):

The enzyme immunoassay (EIA) uses the catalytic properties of enzymes to detect and quantitate immunological reactions. In practice, enzyme-labeled antibodies or antigens (i.e., conjugate) are first allowed to react with ligand and enzyme substrate is subsequent added. Measurement of the resultant decrease in substrate concentration or increase in product concentration is then used either to detect or quantitate the antigen antibody reaction. The product formed is coloured and hence it can be quantified colorimetrically or spectrophotometrically. The intensity of colour can be used to quantify the patient sample. Alkaline phosphatase and horse radish peroxidase enzyme labels predominate in

coupling procedures using bifunctional reagents (e.g., glutaraldehyde)[24]. A heterogeneous enzyme linked immunosorbent assay (ELISA) and a homogeneous enzyme multiplied immunoassay technique (EMIT) are the most widely EIA techniques used in clinical analyses[25,26]. These assays that produce compounds that can be monitored photometrically are very popular. because compact, high performance photometers are now available that are versatile, reliable, simple to operate and relatively inexpensive [27].

1.3.1.2. Fluorescence immunoassay:

Fluorescence is the property of certain molecules to adsorb light at one wavelength and emit light at a longer wavelength, when a photon strikes a molecule, some of the electrons absorb the energy and become excited to a higher-energy state. These excited molecules are unstable and quickly revert to their low-energy ground state, which in the case of rigid molecules may be accompanied by the release of energy in the form of visible light. The emission is termed fluorescence (or occasionally phosphorescence). The structurally rigid molecules capable of fluorescence are called fluorophores. The emitted light has a lower energy (and different colour) than the absorbed light[28].

1.3.2.3. Chemiluminescence immunoassay:

Chemiluminescence is the name given to light emission produced during a chemical reaction. Isoluminol and acridinium esters are the most important examples of chemiluminescent labels used in chemiluminescence immunoassay.

Oxidation of isoluminol by hydrogen peroxide in the presence of a catalyst (e.g.. microperoxidase) produces a relatively long-lived light emission at 4.5 nm and oxidation of an acridinium ester by alkaline hydrogen peroxide in the presence of a detergent (e.g., Triton X-lOO) produces a rapid flash of light at 429 nm. Acridinim esters are high specific activity labels that can be used to label both antibodies and haptens[29].

The immunochemiluminometric assay (ICMA) is the most widely technique used in clinical laboratories. The principle that underlie ICMA and IRMA are the same, except the signals detected differ. In IRMAs the reporter antiserum is labeled with radioiodine, whereas in ICMA is coupled to a chemical reagent that emits a particular wavelength of light when activated. ICMA most commonly use acridinium esters, although other luminescent compounds such as luminol and isoluminol, have been used successfully[29,30, 31,]. The signal generated in ICMA is increased in a few seconds with a luminometer, shortening the time required for the procedure. ICMA is at least as sensitive as IRMAs have more rapid turnaround time, and avoid both the hazard of radiation exposure and the cost of radioisotope disposal. Both types of immunometric assays require immobilization of a solid phase matrix of capture antibodies in sufficient excess to bind all the analyte in an unknown sample.

1.3.2. Isotopic techniques:

The two major types of isotopic techniques that are used in these immuno-chemical assays are classified by radioimmunoassay (RIA) and immuno-radiometric assay (IRMA)[27].

1.3.2.1. Radioimmunoassay (RIA):

Radioimmunoassay is a valid microanalytical technique used for the determination of the concentration of virtually any biologically active substance in biological fluids.

Yalow and Berson (1960)[33], who developed the forerunner of todays RIA procedures by publishing their classical paper on insulin radioimmunoassay however, it is fair to trace back the history of the RIA to the work of Collip and Anderson (1934)[34] who provided some basic principles of radioimmunoassay.

1.3.2.1.1. The advantage of RIA as a microanalytical technique:

The advantage of RIA as a microanalytical technique is their extreme sensitivity, simplicity and specificity which make RIA technique as the method of choice for such measurement.

a. Sensitivity:

This may be defined as the least amount of the material which could be detected by the technique it can be differentiated from zero point (no

material). It is well established that RIA has the capability to measure substance at concentrations as low as one picogram (10^{-12}g) per ml. In general RIA technique are three to six times more sensitive than most clinical techniques such as UltraViolet spectrophotometery (U.V) [34].

b- Precision:

It refers to the reproducibility of the results, i.e the consistency of the results obtained when the test is carried out several times on the same sample under the same conditions (Inter-assay precision). It also refers to the consistency of the results when replicates of the same sample are included in one assay (intra–assay precision). In this case the agreement between replicates is an indication of the precision of the assay[36].

c- Specificity:

Specificity can be defined as the ability of the antibody to recognize an antigen even when the antigen is measured in a medium of similar substances. Therefore, theoretically it is possible to measure a substance of interest in serum, plasma, urine, tissue or whole blood, in the presence of many compounds of similar structure, without having interference with the substance to be measured by this concept RIA techniques are extremely specific[37].

d-Accuracy:

It is defined as the extent to which the value obtained in an assay corresponds to the true value. The accuracy of the technique can be examined by determining of the recovery of added substance (Identical to the one assayed) in the assay. The results are expressed in terms of percent of that added substance recovered [38].

In order to measure very low concentrations of compounds with precision the radioactivity labelled trace r is necessarily required at high specific activity. In case of proteins and polypeptides this is most readily accomplished by radioiodination with radionuclides of iodine, notably I^{125} and that is also used in the preparation of labelled tracers for the measurement by radioimmunoassay of non proteins, particularly small molecules such as steroids and some druges[38].

1.3.2.1.2. Principle of radioimmunoassay:

The basic principle of RIA was the topic of many published in text books and various articles[35,37, 38, 39,]. The reagents required for RIA include:

1- The substance to be measured or detected, eg. Hormone, vitamin, enzyme, protein or drug, etc. this is commonly named the antigen (Ag) or the analyte.

2- The substance labelled with radioisotope. This is commonly called the labelled antigen (Ag*) and was used as labelled tracer in immunoassays.

3- The specific antibody (Ab) produced against the antigen which interest in measuring .

4- The quality controls are identical to the non- labelled antigen and with known concentration, its inclusion in the assay assures reproducibility of results.

5- The series of standards of various concentration prepared from chemically pure substances identical to the antigen or the analyte in the specimen.

6- Reagents for the separation of the substance bound to the antibody from the unbound substance.

Radioimmunoassay works according to the following principles:

The antigen in the specimen and labelled antigen will compete for the limited binding sites on the antibody. After an equilibrium time, a certain amount of the specimen antigen will be bound to the antibody and a certain amount of the labelled antigen will also be bound to the antibody. The more antigen there is in the specimen, the less radioactive antigen will be bound to the antibody. Therefore, a leftover antigen (either lablelled or unlabelled will be unbound to the antiboddy. The next step is to separate and determine the amount of antigen that remained unbound (Free) and the amount that become bound to the antibody

(B). From this information, one can determine from a standard curve how much antigen of interest there was in the specimen.

The following equation explain the basic idea of RIA technique:

$$\text{Antigen (Ag)} + \text{Antibody (Ab)} \rightleftharpoons \text{Ag-Ab} + \text{Leftover Ag.}$$

$$\text{Radiolabelled Antigen (Ag*)} + \text{Antibody (Ab)} \rightleftharpoons \text{Ag*} - \text{Ab} + \text{Leftover Ag*}$$

The summations of the two equations will give the following eqution:

$$\text{Ag*} + \text{Ag} + \text{Ab} \rightleftharpoons \text{Ag-Ab} + \text{Left over Ag.}$$

$$+\text{Ag*} - \text{Ab} + \text{Left over Ag*}$$

The standard curve is constructed by running the assay with known amounts of purified antigen standards and the percent bound or percent free are plotted on the Y-axis against various concentration of antigen on the X-axis. From the last diagram, it can be seen that if labelled antigen (Ag*) and specific antibody (Ab) are mixed with the specimen antigen (Ag) and allowed to react until they reach equilibration, two complexes and one of the left over antigens are labelled. If the amount of the labelled antigen added is fixed as it is, then by competitive binding the amount of labelled complex (Antigen*- Antibody) formed will be inversely dependent on the amount of unlabelled antigen present[40,41,42].

1.3.2.2. Immunoradiometric Assay (IRMA):

The immunoradiometric assays (IRMAs) are non-competitive immuno-assays. IRMA was described by Miles and Hales (1968)[42]. IRMAs are the method of choice for measuring most hormones, especially peptides and proteins[44]. Unlike RIAs, IRMAs use saturating concentrations of two or more antisera that recognize non-competitive epitopes present in the analyte. IRMAs require immobilization to a solid phase matrix of capture antibodies in sufficient excess to bind all the analyte in an unknown sample[27,45], reported that in a typical non competitive assay for an antigen, a capture antibody is first passively adsorbed or covalently bound to the surface of a solid phase. Various sequences in which the capture antibody can be attached were reported. The simplest involves direct attachment to the solid phase. However, this can lead to some loss of antibody binding capacity because of steric factors or attachment of the antibody via its binding region. To protect the binding properties of the antibody, more complex sequences have been devised. For example, the solid phase support can be coated with an antispecies antibody and then the antispecies antibody can be used to immobilize the capture antibody via an antigen, antibody reaction[27]. In another report Serge and Brown[44], stated that the antigen can also be biotinylated and then bound to a matrix to which avidin has been covalently coupled, or it can be bound to tile matrix by a second antibody that recognizes the immunoglobulins from the animal species that generates the analyte specific, first antiserum

In the second stage of the assay, the antigen from the sample is allowed to react with the solid-phase capture antibody, other proteins are washed away. A second antiserum containing antibodies (reporter antiserum or conjugate) that do not compete with the first antiserum for binding to the antigen through second and distinct epitope is labelled to high specific-activity and is then added in solution to the assay. The reporter-antiserum binds to the analyte, which has been immobilized by its binding to the first antiserum. The unknown analyte or standard functions as a bridge from the solid-phase matrix to the reporter antiserum. Since the analyte is the limiting factor in the reaction, the amount of soluble, reporter antiserum bound to the solid-phase matrix is a function of the analyte concentration. After washing again, the bound label is determined in a gamma counter. At the same time a standard curve is prepared with the standards. The analyte concentrations in the samples are then obtained by interpolation from the curve. The concentration of the analyte is directly proportional to the radioactivity[46]

Solid phase - Ab + Ag $\rightleftharpoons$ Solid phase - Ab Ag

+

Excess Ab*

$\Downarrow$

Solid phase - Ab Ag Ab* ± Free Ab*

In **non-competitive assays (IRMA), the capture and labelled antibody can be** either polyclonal or monoclonal. If monoclonal antibodies having specificity for distinct epitopes are used, it is possible to incubate the sample and conjugate

(reporter antibody) simultaneously with the capture antibody, thus simplifying the assay protocol[27]. From mentioned before, RIA method is a good and available technique to determine AFP antigen. The components of AFP-RIA system are anti-AFP polyclonal antibody, radiolabelled antigen, standards, quality control, and separating agents.

1.4. Production of polyclonal anti-AFP antibody:

The requirements are for an antibody having the requisite to give continuity of use for a reasonable time span. If these requirements can be clearly defined for a given purpose and the demand is considerable then may be desirable to produce sufficient of a single antibody for widespread distribution[47,48, 49]. There are certain choices of approach that can be made in raising antibody of suitable quality; however, it should be stressed that all antibody should be critically assessed and it should not be assumed that the pattern of antibody production obtained with one animal will necessarly be repeated with subsequent individuals.

1.4.1. Choice of animal species:

This is largely dictated by practical considerations, if large volumes of antibody are required, then large animal species such as sheep or goats are useful; however, many laboratories do not have the facilities required for such big animals. Rabbits are of a convenient size for a simple animal house, are

easily bleed from marginal ear vein and yield sufficient antiserum, which contains antibody for most individual, as opposed to commercial, purposes. New Zeoland white rabbits have generally been found to be a suitable breed, using either a particular strain known to be good antiserum producers, or randomly breed animals[50].

1.4.2. Immunization:

In practice it is essential to use some forms of adjuvant when raising antibodies to enhance the immune system of the immunized animal although an adjuvant effect can be obtained by adsorption of the immunogen on to particales (e.g carbon, alumina), or trapped in a polymer (e.g polyacrylamide), these materials are not always satisfactory and a water in oil emulsion is best employed. Indeed, these particular and polymer preparation are best used in conjunction with Freund's. The immunogen is dissolved in saline and added to two volumes of adjuvant, and the mixture is emulsified. Emulsification can be accomplished using high-speed mechanical homogenization, ultrasonication, or more simply by repeatedly forcing the mixture between two syringes connected by a narrow-bore hub. The emulsion should be stable if a drop is placed on to the surface of cold water it should remain cohesive[52]. The primary injection is given in Freund's complete adjuvant (containing mycobacterium); subsequent immunizations are given in the Freund's incomplete adjuvant.

In theory, the dose of antigen injected should be low, so as to maximize the affinity of the antibodies obtained. In practical terms, about 100μg of immunogen are injected per animal. The currently perferred route of immunization is intradermal at multiple sites, which gives a very rapid response. However, some workers find that such a procedure gives no better results than subcutaneous injections at four sites: two suprascapula and two inside the thighs or over the pelvic region[47,52]. After primary injection the antiserum titre rises slowly and reaches a plateau at 4-6 weeks, after which a variable slow decline occures. Subsequent injections, which should not be given until this plateau has been reached, produce a more rapid response, which reaches its maximum after 7-14 days and may then either fall off quickly or show little change during subsequent weeks. The secondary response may produce an increase in titre whose magnitude may vary, generally from two folds to ten folds over the peak given by the primary response. Subsequent boosters may or may not produce improvements in titre.

The affinity of antibody generally increases following the primary inoculation although this is not invariable and avidity may fall off or increase suddenly and unaccountably during a series of boosters. There is evidence that high avidity is favoured by spacing boosters widely (3-6 months is recommended) and there are examples of good avidity returning when an animal has been rested for periods of several months after a series of a shorter-spaced

injections. The specificity of the antiserum obtained is improved particularly for protein antigens, if a highly purified preparation is used as the immunogen. There is also some evidence that specificity improves during immunization, although this is by no mean universally observed[49].

1.5. Radiolabelled antigens as tracer:

Since the introduction of radioimmunoassay, there has been a need for high specific activity tracers in order to measure the concentration of analyte efficiently in the picomole range. The most commonly used radiolabele is ^{125}I and the mechanism of iodination must be considered if adequate preparations of traces containing this radiolabele are to be made.

Between pH 7 and 8, the ortho position in aromatic ring of tyrosine moiety of peptides and proteins is activated for electrophilic attack, owing to the electron-donation effect of the neighboring hydroxyl group. Similarly, at pH 9 the two nitrogen atoms in imidazole moiety of histidine have an electron-donating effect on their mutul neighboring carbon atom. Iodine, in the form of iodous ion (I^+), acts as the electrophilic agent to give the radiolabelleled tracers[53, 54,55].

1.6. Standards for radioimmunoassays:

Assay standards can be considered at two levels: working or in-house standards, used as day to day standards for dose-response curves; and national or international reference preparations (primary standards). As a general principle, the standard preparation should be as similar as possible to the substance being measured in unknown samples. In the case of many small molecules, the chemical identity of the compound is known and pure synthetic material is available as in T_3, T_4. For more complex analytes, particularly proteins, the detailed structure may not be known and endogenous material itself may not be completely homogeneous. Obviously the preparation and provision of standards for this latter group of analytes may pose considerable problems. In addition to the problem of chemical identity, another feature of primary standards is their stability. They must be able to be stored for long time periods and be transportable to individual laboratories[56].

In case of AFP-RIA system, the cord blood has a high concentration of AFP-antigen, so from serum of cord blood, AFP standards can prepare after purification and dilution with a suitable matrix.

1.7. Separation system for bound and free fraction

Free antigen may be separated from antibody-bound antigen by physco-chemical systems (e.g. electrophoresis, chromatography, adsorption, etc.). The

separation can carried out using solid-phase techniques (using antibody linked to some forms of solid matrix) or by means of second antibody precipitation, system, where the antibody γ-globulin complexed to the labelled antigen is precipitated by anti-globulin raised in a second animal species. In some cases polyethylene glycol (PEG) was used with second antibody precipitation technique.

1.7.1. Physico-Chemical methods for separating bound from free antigen:-

Since antibody gamma globulin are large proteins than many analytes measured by immunoassay, an antigen-antibody complex will after approximate to γ-globulin in its physico-chemical behaviour. In some systems used to separate bound antigen, both the nature of the antigen and the number of antigen molecules in the complex may be expected to affect the behaviour of the bound moiety. However, it should not be assumed that the bound component of all systems will behave in the same way. In each system the behaviour of the bound and free antigen must be investigated [57].

1.7.1.1. Separation by electrophoresis:

In this technique the separation of bound and free antigen may be achieved by means of electrophoresis on paper, cellulose acetate, or in starch or polyacrylamide gel [58].

1.7.1.2. Adsorption systems:

Adsorption system employing paper chromatography played an important role in the first years of the development of the radioimmunoassay. Activated charcoal is widely used as separation system, particularly for hapten immunoassays. Excellent separation systems, however, may be devised if the charcoal is first treated with dextran and / or protein. The variables which must be considered are nature and amount of charcoal and volume in which it is added it the incubates nature and amount of coating substances, ionic species concentration and pH of buffer used, and the time course of the adsorption[59,60].

1.7.1.3. Ion-exchange systems:

Ion-exchange resins may be used for immunoassay separation systems in much the same way as are absorbants. Again, a considerable investigation is necessary in developing this method for a particular purpose and has not been widely used probably because it is difficult to avoid interference due to changes in plasma protein concentration in assay incubates [60, 61].

1.7.1.4. Methods based on molecular size:

Two methods that exploit the disparity in size of the smaller free antigen and the bound complex are worthy mention. Ultracentrifugation has been used as an independent method to confirm the presence of antibodies to insulin. Gel filtration, e.g. on sephadex, offers a general method for separation free from

antibody bound antigen providing the antigen has a relative molecular mass of less than about 100,000 [62].

1.7.1.5. Solvent and salt precipitation systems:

These systems have generally attempted to precipitate the bound fraction while leaving the unbound antigen in solution. Early the sodium sulphate and ether were used with insulin.[63] used salts and ethanol for TSH. All such methods require careful optimization and have only limited applicability[64].

1.7.2. The double antibody method:

This is a general method and depends upon the finding that the antigenic determinants on an antibody are separate from its antigen-binding sites. An antibody molecule may, therefore form a complex with its antigen and then itself be complexed to a second antibody. In highly sensitive immunoassays the primary antigen- antibody complex is too dilute to be precipitated; however, if carrier non-immune serum belonging to the same species as the first antibody is added and this is followed by antiserum raised in a second animal species to the γ-globulin of the first antibody, a sizeable lattice can be built up and the whole, including the labelled antigen bound to the first antibody, can be precipitated. It is necessary, therefore, to add carrier non-immuno serum from the same species [65].

1.7.2.1 Accelerated double antibody system:

At the relatively high concentration of the double antibody reagent used the immunological reaction between γ-globulin of the first antibody and the second antibody is rapid, and the delay involved in this separation method is to allow a full precipitation to occur. This second precipitation stage of the antigen antibody complexes can be accelerated by the addition of low concentrations of various materials. Such as polyethylene glycol (PEG). Ammonium sulphate and dextran were the first investigation of accelerator of the double antibody system for RIA assays. Recently, the more widly used PEG system have been subjected to detailed investigation [66].

1.7.2.2. Pre –precipitated double antibody systems:

In this approach the first antibody is precipitated by suitable anti-γ-globulin in such away as to preserve its immunoreactivity. This is accomplished by using carefully tested dilution of both first and second antibody, carrier non-immune serum for the first anti serum, or both antisera, being included. A suspension of this precipitate is added to immunoassay incubates which may or may not require continuous agitation. Separation of the bound component is then achieved by centrifugation. This method may be economical in its use of second antibody since, in contrast to the ordinary double antibody method, little or no carrier first serum may be needed. On the other hand, there may be considerable

waste of first antibody since some of its activity may be lost, presumably by trapping within the precipitate[67,68].

1.7.3. Solid-phase antibody systems:

There are two types of solid-phase systems can be recognized, those using particulate matrices (e.g. cellulose, agarose) and those using a continuos surface (e.g. tubes, beads, discs). All solid phase systems have low non specific binding[69,70].

1.7.3.1. Surface solid-phase systems:

The use of an antibody fixed onto the surface of an immunoassay reaction tube provides conceptually the simplest assay system of all; sample and tracers are added to the antibody-coated tube. Incubated for a fixed time, and the bound and free fractions of antigen are separated by pouring the contents out of the tube, which can be washed, if required. Antibody-coated tubes suffer from the draw backs of all solid-phase antibody systems. i.e. Potential losses of sensitivity and antibody titre when large analytes are measured, but have the advantages of simplicity and an avoidance of centrifugation [71,72].

1.7.3.2. Particular solid – phase systems:

Sephadex, cellulose, agarose or sephacryl were used as the support medium. Avariety of methods have based on using sephadex and sepharose, for coupling

of antibody, including diazotized amino-cellulose, the imidazoloyl carboamate, cyanogen bromide activation of cellulose, derivative of cellulose, and periodate oxidation of cellulose or sephacryl. Although widely investigated and technically simple to carry out, the cyanogen bromide activation involves the use of highly toxic (and explosive) material and is not, therefore, suitable for large scale preparations [73,74,75].

1.8. Radiolabelling methods:

A number of radioiodination methods for proteins have been reported. Notably those include: chloramine-T[75], lactoperoxidase[76], iodogen[77], electrolytic iodination[78,79] and indirect conjugation[80]. However, each of the above methods suffers from certain disadvantages [80].

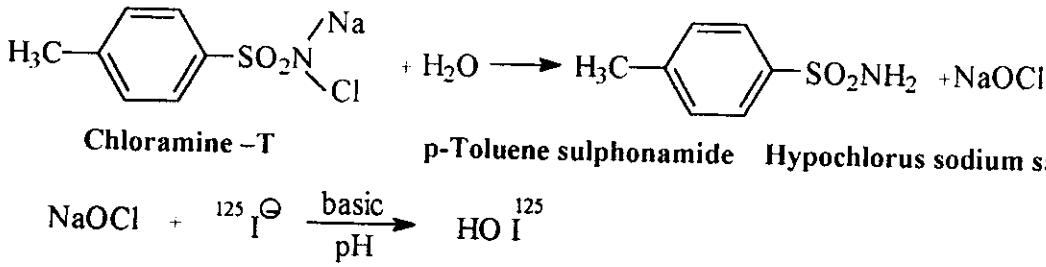
The interest shown for the element iodine in the field of nuclear medicine can be attributed to the following facts (i) iodine is a biologically important element, (ii) chemically, it can take different forms: metal, anion or cation (iii) physically, it has many radioactive isotopes [81].

1.8.1. Radionuclide selection for labelling:

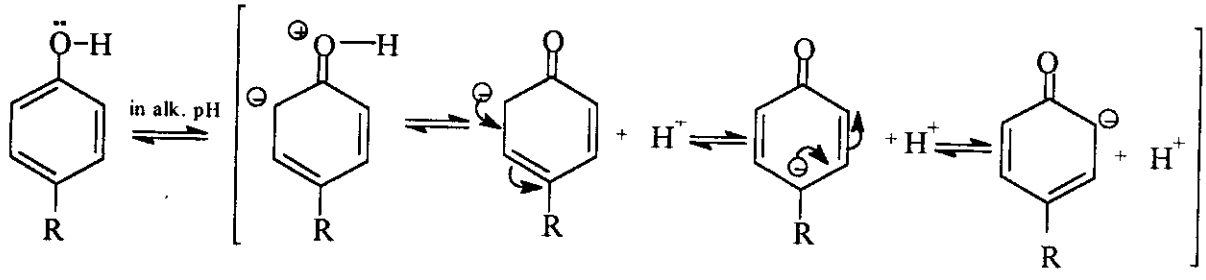
Gamma-emitting radionuclides have many advantages when used in the preparation of labelled tracers for competitive protein binding and radioimmuno-assay procedures. Probably the greatest advantage is the ease with which gamma

emitters can be counted. no special sample preparation is required and this can be particularly important when large numbers of samples are involved as commonly in the case of radioimmunoassay. But beta-emitting radionuclides require liquid scintillation counting system which are expensive in terms of scintillation fluids and often require somewhat time consuming and tedious sample preparation procedures. Radioiodine particularly convenient for the preparation of protein tracers as it can be readily substituted into tyrosine residues of polypeptides. Two gamma–emitting radioisotopes of iodine are widely available, ^{125}I and ^{131}I. In addition to ease of counting, these offer the great advantage, over the common β-emitting isotopes ^{3}H and ^{14}C, of a much higher specific activity, one gram atom of ^{125}I gives approximately 100 times the count rate produced by one gram atom of ^{3}H and about 35,000 times the count rate produced by one gram atom of ^{14}C[82,83,84]. The radioiodinated compounds at present constitute the tracers of choice in RIA techniques where high specific activities are required. ^{125}I and ^{131}I have been widely used for tracers preparations for radioimmunoassay systems. ^{131}I has the shorter half –life,8 days compared to 60 days for ^{125}I and consequently has a higher specific activity at 100% isotopic abundance. However, ^{131}I is available at only about 20% isotopic abundance due to a major contaminant of ^{127}I. Both isotopes are usually counted in well-type sodium iodide crystal scintillation counters in which the counting efficiency for ^{125}I is about twice that for ^{131}I. Because of the short half-life, the count rate for ^{131}I decreases more rapidly with time than for ^{125}I and decay corrections also have to be made

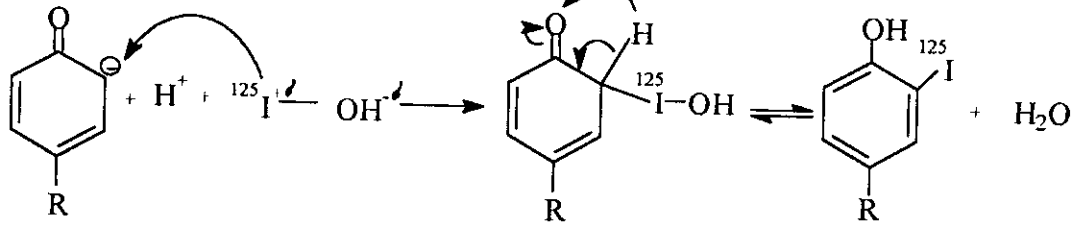
Fig(1): Radioiodination of proteins by electrophilic substitution mechanism:



Chloramine –T **p-Toluene sulphonamide** **Hypochlorus sodium salt.**



Tyrosine residue **Intermediate**



Where R = $-(CH_2)_nCHNHCOOH$

1.8.4. Choice of radioiodination method:

The choice of a radioiodination method depends on several factors listed here in descending order of importance:

Preservation of biological activity of the original molecules (immuno-reactivity integrity), sensitivity of the molecule to oxidation damage, the desired specific activity, and the labelling kinetics. There are several methods for the direct iodination of proteins and peptides (i) the iodine monochloride method (ii)

the chloramine-T method (iii) Alternative chemical oxidation methods (iv) insoluble oxidizing agent, the iodogen method (v) electrolytic iodination, (vi) enzymatic iodination.

1.8.4.1. Iodine monochloride method:

Iodine monochloride is equilibrated with radioiodide which is then reacted with the protein to the labelled example, the radioiodination of insulin with ^{131}I to high specific activity for use as a tracer in the RIA. The substitution of iodine into protein can be precisely control in the iodine monochloride method by using a fixed mass of the reagent [87,88].

Disadvantages of this method are that the protein is exposed to the potentially harmful radioiodide solution for a considerable time and that non-radioactive iodine is inevitably incoported into the protein, lowering the specific activity that can be obtained. In general, this method has not been widely used for the preparation of high specific activity tracers for radioimmunoassays.

1.8.4.2. Chloramine-T method:

This is the most widely used method for the radioiodination of small mass of protein to high specific radioactivites for use as tracers in radioimmunoassay. The molecular structure of chloramine –T is represented as following:

1.8.4.3. Alternative chemical oxidation methods:

In order to overcome possible problems of protein damage caused specifically by chloramine-T, several alternate oxidation methods have been proposed which avoid this reagent [88]. From these methods, sodium hypochlorite and N-bromosuccinimide.

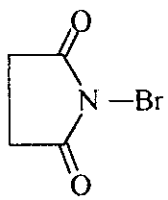
1.8.4.3.1. sodium hypochlorite used as an oxidizing agent [90]:

$Na^{125}I$ buffered with phosphate buffer to pH 7.5 then reacted with the antigen in the presence of sodium hypochlorite solution. The optimum amount of sodium hypochlorite for each protein to be labelled has to be determined separately-greater and lesser amounts of oxidizing agent resulting in reduced specific activities of the labelled products. The resulting labelled antigens are reported to show great stability when used as tracers in radioimmunoassay. However, this method also has the potential disadvantage of all direct chemical methods, namely, exposure of the protein to possible harmful substances. This method avoids exposure of protein to chloramine-T.

1.8.4.3.2. N-bromosuccinimide method:

The use of the mild oxidizing agent N-bromosuccinimide for protein radioiodination avoids exposure of proteins to harsh oxidizing agents like chloramine-T. This method which is claimed to be superior to many other particular methods by yielding tracers with low non-specific binding characteristics. However, it would appear that in these studies the

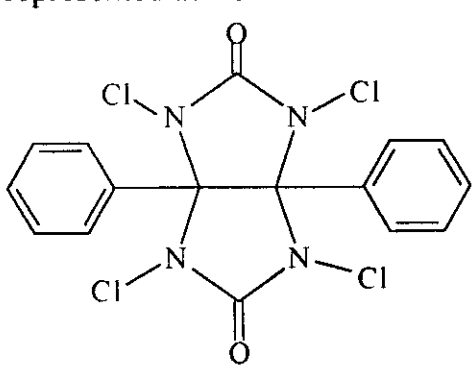
N-bromosuccinimide method has been more carefully optimized, for example with respect to minimizing oxidant concentration, than the other methods used for comparison [91].



N-bromosuccinimide

1.8.4.4. Iodogen method:

In an attempt to avoid the harmful effecte of soluble oxidizing agents on proteins, Franker and Speck investigated the use of the water-insoluble chloro-amide 1,3,4,6-tetrachloro3α,6α-diphenylglycoluril (Iodogen). The molecular structure of iodogen is represented as Follow:



1,3,4,6-tetrachloro3α,6α-diphenyl glycoluril (Iodogen).

This may coated onto the surface of the reaction vessel and the iodination affected by the addition of $Na^{125}I$ and protein solution to this vessel. Although this method has various potential disadvantages including the relatively long reaction time (up to 35 min) during which the protein being labelled is in dilute

carrier free solution, superior results to the chloramine-T method have been claimed.

1.8.4.5. Electrolytic iodination method:

As an alternative to chemical oxidation methods of protein iodination, Pennisi and Rosa[78] reported the use of constant currents electrolysis to convert iodide to iodine as an efficient and mild procedure for the iodination of insulin, where a concentration of $8.5x10^{-6}$ M in 0.9% NaCl solution is mixed with $Na^{125}I$ and electrolyzed for about 40 minutes, with a current of 6-7 μA. High labelling yield (80-90%) can be achieved, with consequent high specific radioactivities. The resulting products retain high levels of immunological and biological activity One of the advantages of this method is that proteins are not exposed either to oxidizing or reducing agents [78].

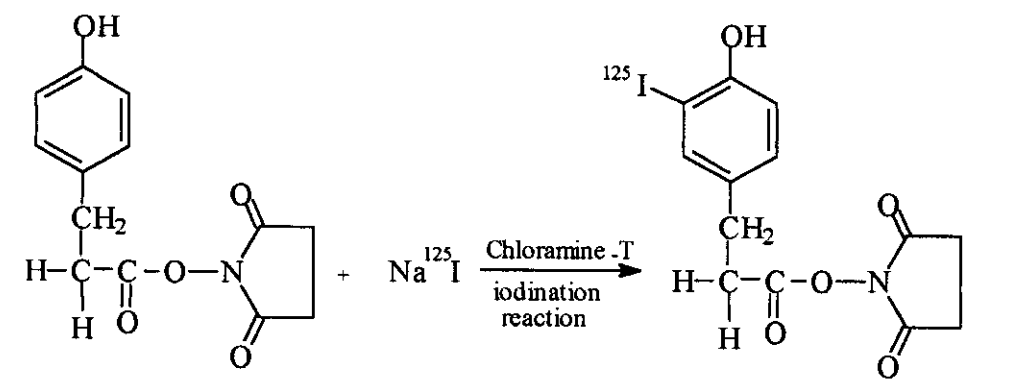
However potentially damaging radioiodide solution which they are maintained in contact with proteins intended to be labelled for a considerable period of time which may readily lead to the denaturation of some unstable protein. The technique is more complex to perform than simple chemical iodination, and specialized equipment in the form of the micro-electrophoretic cell is required [79].

1.8.4.6. Enzymatic iodination method:

A gentle method for the radioiodination of proteins using the enzyme lactoperoxidase to catalyse the oxidation of iodide in the presence of very small amount of hydrogen peroxide was first described by Marchalonis (1969)[76] for the iodination of immunoglobulins. The reaction is initiated by the addition of hydrogen peroxide and is maintained by further additions of hydrogen peroxide at intervals. At the end of the reaction period the reaction is terminated either by the addition of cysteine which quenches the lactoperoxidase catalyzed iodination, or by dilution, and the products of the iodination reaction are separated by gel filtration. The enzymatic radioiodination of proteins has been found to minimize damage to labelled proteins, maintaining their structural integrity, and thus resulting in preparations with a high retention of immunological and other biological activities. It avoids the exposure of the protein to a strong oxidizing and reducing agents although proteins are exposed to the potentially harmful radioiodide solutions for considerable period of time (10-30 minutes) and the hydrogen peroxide is used as a potent oxidizing agent. The problem of the relatively low yield of the iodination reaction may make it difficult to obtain radioiodinated proteins of high specific radioactivity [90, 91].

1.8.5. Conjugation labelled methods for the iodination of proteins and peptides (Bolton and Hunter)method [56].

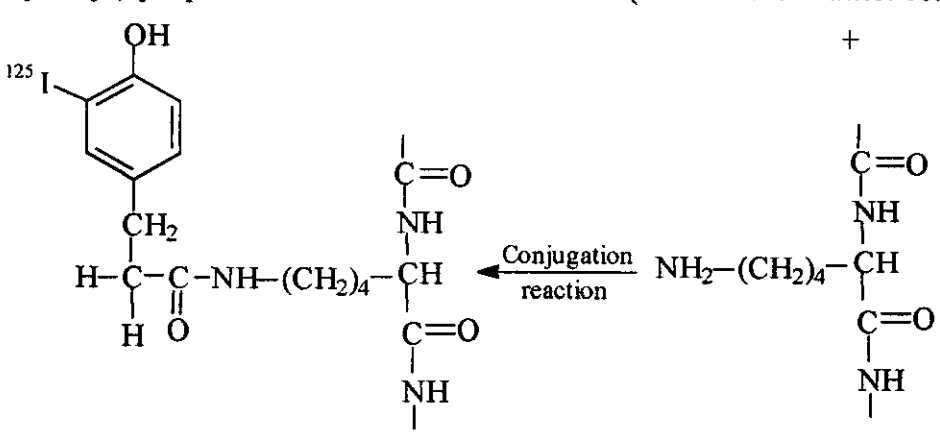
The N-succinimidly group of this reagent condenses with free amino groups of proteins or peptides to form a conjugate in which a radioiodinated phenyl group is covalently linked via an amide bond to the protein. The conjugation labelling procedure requires the initial radioiodination of N-succinimidyl 3-(4-hydroxy phenyl) propionate by the chloramine –T reaction to give Bolton and Hunter reagent (Fig. 2)[92].



N-succinimidyl 3-(4-hydroxy phenyl) propionate

Iodinated propionate (Bolton and Hunter reagent)

+



(The final labelled compound)

Σ-Amino group of lysine in protein to be labelled

Fig (2): conjugation labelled method for the iodination of proteins and peptides.

Under aqueous conditions this material is rapidly hydrolyzed to 3-(4-hydroxy phenyl) propionic acid and the iodination reaction must therefore be carried out as rapidly as possible to minimize this hydrolysis. The labelled material is then extracted from the aqueous phase into an organic solvent and thus separated from the other reactants of the chloramine-T reaction. Labelled material can either be used directly after removal of the organic solvent or can be further purified and the monoiodinated and diiodinated species separated by thin-layer chromatography or, by HPLC before use the second stage of the labelled procedure which involves the reaction of the radioiodinated succin-imidyl propionate with the protein to be labelled. The conjugation reaction takes place under mildly alkaline conditions, (pH 8-8.5). The conjugation reactions is markedly concentration dependent and thus requires the use of the protein dissolved in the minimum volume that can be handled and if the reaction vessel is a narrow pointed conical tube it must be as little as 10 μl [92].

The conjugation labelling method was specifically developed to overcome problems of iodination damage associated with exposure of proteins to oxidizing and reducing agents and radioiodide solutions. There is a minimum chemical attack on the protein. The method offers the additional potential advantage of substituting different amino acid residues. Substituting different amino acid residues of the protein offers more advantages than other iodination methods.[93,94]. The dissadvantage of this method is that it is technically more

complex to perform and requiring more manipulation of radioactive material with the associated hazards. Because it is a two stage reaction, the overall iodination yields are lower than that of direct iodination methods which resulting in a smaller proportion of the original radioiodide used.

1.8.6. Purification of radiolabelled protein from unreacted radioiodide:

This relatively simple biochemical fraction requires the separation of small molecule, $Na^{125}I$ from a larger peptide or protein. The most straight forward method is to use gel filtration. A small (0.9 x12 cm) column of Sephadex G-25 or G50 is generally adequate for most proteins, although peptides of low molecular weight may require Sephadex G-10 or G-15 or a suitable grade of bio gel poly acrylamide gel filtration medium to effect the separation. If Sephadex column is used it must be remembered that some molecules are strongly adsorbed and elute rather more slowly than would be expected for their molecular size. An example is iodinated oxytocin which elutes from Sephadex column after the iodide peak. The gel filtration medium is equilibrated in phosphate –carrier protein buffer and packed into a column (0.9x12cm). The column is saturated with protein before use to minimize the adsorption of the very low concentrations of labelled protein to be separated. This saturation is accomplished by adsorption of the very low concentrations of labelled protein to be separated. This saturation is accomplished by passing 0.5 ml of a suitable carrier protein solution (for example 100 mg/ml solution of bovine serum albumin through the column.

The iodination reaction mixture is quantitatively transferred to the prepared column and eluted with phosphate–carrier protein buffer. One ml fraction are collected into polystyrene tubes of the same dimensions as the iodination reaction tube. The column is run until both the protein and the [^{125}I] iodide peaks have been eluted, and the radioactivity in all fractions is measured [96].

1.8.7. Assessment of the radioiodinated tracer.

Several parameters ought to be credited to assess tracers stability for RIA. They are radiochemical purity, immunoreactivity, specific activity and stability of the preparation.

1.8.7.1. Radiochemical purity:

The quality of a radioiodinated preparation is normally at its highest value immediately following its preparation and purification. Because of self decomposition, however, this quality is not maintained on storage. The carbon-iodine bond is relatively weak, compared with the carbon-hydrogen bond, the main impurities formed in the self –decomposition of iodine labelled compounds is inorganic iodine. Chemical decomposition, self radiolysis and radiolysis of solutions of radioiodine compounds by external gamma irradiation, all yield radioiodine ions as radiochemical impurity [97,98].

1.8.7.2. Immunoreactivity:

It is too necessary for RIA tracers to have an immunoreactivity identical to the analytes being measured. Nevertheless, some tracers damage can generally be tolerated in such assay. Even slight configuration changes in the labelled material could result in a loss of affinity. Any loss in affinity tracers to antibody compared with unlabelled antigen will result in some loss in assay sensitivity. It is preferable to use, as far as possible, a tracers with minimally damaged and bearing similar immunoreactivity to unlabelled antigen. Possible differences in the immunoreactivity of labelled antigen can results from a batch to batch which may generally be attributed to the following reasons [94,99]:

a) The degree of iodine substitution in the antigen molecule:

The degree of radioiodination of a given antigen i.e. the number of radioactive iodine atoms per molecule of antigen (specific activity) has an effect on its immunoreactivity (Affinity for binding to antibody). In general, the greater the degree of iodination, the lesser the immunoreactivity of the antigen [48,84].

b) Chemical damage:

Exposure of the antigen to oxidizing and reducing agents employed in the radioiodination procedure and other impurities might impair the integrity, of the antigen and are potential cause of less immunological activity[100].

c) Denturation of protein caused by storage and handling

It is generally essential that protein preparations which are to be used for labelling are maintained in a carrier-free state (that is not mixed with carrier proteins such as albumin) and they are present in highly dilute solutions. Many proteins are unstable and under these conditions may resulting in denaturation which is revealed, after radioiodination, as damaged tracer. Such effects can be minimized by the addition of a carrier protein (for example. Albumin. Animal serum) as soon as possible after the protein has been labelled [96].

d) Radiation damage:

This refers to structural alteration of the protein caused by exposure to high levels of radiation during or after iodination. Early workers used high levels of radioactivity (Yellow and Berson used 30–80 mCi ^{131}I for the iodination of insulin in their early radioimmunoassay), but iodination methods currently in use require much lower levels of radioactivity than formerly and those are probably insufficient to cause radiation damag [37,97].

1.8.7.3. Specific activity (SA):

Bolton, 1985 has reported that in order to measure very low concentrations of compounds, precisely by RIA, the radioactivity labelled-tracer is necessarily needed with high SA[84]. The only advantage of increasing tracer SA in RIA is to reduce the time of counting. Furthermore, higher SA preparations are generally

less stable than those of lower SA. The extent of chemical substitution with radioactive iodine may affect the tracer affinity, and in general, such affinity would be expected to diminish as more of such groups are built into the antigen. Therefore, the optimum SA for a particular tracer should be the minimum compatible with desired assay sensitivity and the availability of counting time [85].

Determination of specific activity:

Determination of specific radioactivity of the tracer is necessary, for a tracer, SA can be calculated indirectly by one of three methods as follows: The first is called "analysis of the reaction mixture"[49,101]. The method is based primarly on the original chloramine-T work. Such a purely radiochemical method does not take into acount any possible loss in immunoreactivity of the tracer.

$$\text{Specific activity (SA)} = \frac{\text{Actual activity } (\mu Ci) \times \text{yield } \%}{\text{Protein content } (\mu g)}$$

The second is called "Self-displacement:" or "auto displacement" method. The self-displacement method is the common procedure for SA determination and is widely used by RIA workers, where a "tracer curve" is constructed by incubation of increasing amounts of labelled antigen with a constant amount of antibody under conditions similar to those used in regular RIA. Estimation of SA is carried out by comparing the ratio of bound to free tracer with B/F for a normal RIA " standard curves[102].

Third method called, "isotopic dilution" which was described by Englebienne & Selgers, 1983[103]. Since it is based on radioimmunological binding

, this method shares the same limitations mentioned in the above two methods and is not widely used.

1.8.7.4. Stability and shelf-life (storage):

The shelf-life of each batch of radioidinated tracer is a necessary measure in order to assess the radioiodination procedure more accurately. This can vary greatly between preparations, even for one radioiodinated antigen being iodinated at intervals, even for a standardization procedure[52]. It has been reported that the quality of radiochemical compound is normally at its highest value, immediately after its preparation and purification, but, because some factors, such as self-decomposition, this quality is not maintained on storage. The labelled compound becomes unstable and decomposes with varying degrees.

The important factors to be considered which might affect shelf-life are 1) Physical half-life, 2) liberation of free iodide, 3) loss of immunoreactivity , 4) formulation which the tracer is present and also, 5) storage temperature. Most workers prefer ^{125}I for radioidination, not only for its moderately longer half-life but also because of the greater radiochemical stability [48, 84]. On the contraery, that, because of the relatively short-life of ^{125}I, new tracers must prepared, at least every two months[104].

1.9. Performance characteristics of the optimized RIA system:

Performance characteristics of the developed RIA systems under investigation were studied in terms of sensitivity, precision, and accuracy.

1.9.1. Sensitivity:

Sensitivity (or " minimal detectable dose") of a radioimmunoassay standard curve has been defined in many ways. However, the usually followed method is to calculate the minimum dose (or smallest value) which can be defined from zero. In this case the standard deviation of the response parameter i.e. counts (20 replicates) of the zero tubes is calculated and the dose at zero standard counts-2SD of zero standard counts or two standard deviation below the counts at maximum binding (100-2SD) of B/B_0 or as the concentration at 95% B/B_0 is calculated as sensitivity. In practice, sensitivity should be a term that combines both precision and slope of the standard curve. The above mentioned method takes the slope as well as precision. Therefore, The second method was applied in the present study for the calculation of the detection limit (or" minimal detectable dose") of the AFP.

1.9.2. Precision:

This is a baseline for long term quality control. The reliability of the locally prepared AFP-RIA system under investigation was assessed by examining its

reproducibility on, internal quality control samples (low. medium and high) to represent range of AFP levels as follows:

Low quality control (L)= 20-30 IU/ml

Medium quality control =105-150 IU/ml

High quality control (H) over 200 IU/ml

Precision investigations were performed in terms of intra assay precision (within-run variability) and inter assay precision (between-run variability)as indicated by % coefficient of variation (%CV).

1.9.2.2. Intra assay precision:

It was determined using 20 replicates of the three samples (low, medium and high) three quality controls throughout a single occasion of a single assay.

1.9.2.2. Inter-assay precision:

It is an index of the ability of the assay to reproduce a result on the same sample from day to day, or the confidence interval about a single result. Inter assay precision was determined using ten replicates of the above three quality controls on ten separate assays at different occasions according to **Garrett and Krouwer,** (1985) [105].

1.9.3. Accuracy:

Investigation of the RIA systems accuracys were done in terms of recovery according to **(Pillai and Bhandarkar, 1998)**[46] and dilution tests **(Perlstein, 1979)**[106].

1.9.3.1. Effect of recovery:

It was done to find out errors arrising out of the differences in the matrix between the sample and standard. A patient samples were assayed by the RIA system under investigation in assays which included 1:1 dilution of the sample with each of standards at low, medium and high levels

The % recovery was calculated as:

$$\% \text{ recovery} = \frac{\text{observed value}}{\text{expected value}} \times 100$$

1.9.3.2. Effect of dilution:

To estimate the dilution effect on the accuracy of a given assay, a patient samples were assayed undiluted and at different doubling dilutions i.e. 1:2, 1:4, 1:8 and 1:16 with zero calibrator and assayed with the assay system of interest according to **Perlstein, 1979** [106].

1.9.4. Validity and comparison with the available commercial kits:

Results from the protocol of local RIA system under investigation were compared with results on the same specimens obtained (with different pathological cases of AFP values) using a known "reference" methods i.e, CIS coated tube RIA kit, NETRIA coated beeds and DPC double antibody RIA kit). Results are analyzed by linear regression analysis and correlation coefficient "r" was determined.