**IMMUNOASSAY:**

**An immunoassay is a test that uses antibody and antigen complexes as a means of generating a measurable result**.

An antibody: antigen complex is also known as an immune-complex. **“Immuno”** refers to an immune response that causes the body to generate antibodies, and **“assay”** refers to a test.

Immunoassays are different from other types of laboratory tests, such as colorimetric tests, because they use antibody: antigen complexes to generate a signal that can be measured. In contrast, most routine clinical chemistry tests utilize chemical reactions between the reagent (a solution of chemicals or other agents) and patient sample to generate a test result.

**Both the presence of antigen or antibodies can be measured e.g,**

* When detecting infection, the presence of antibody against the pathogen is measured.
* For measuring hormones such as insulin, the insulin acts as the antigen.

For numerical results, the response of the fluid being measured must be compared to standards of a known concentration. This is usually done through the plotting of a standard curve on a graph paper, and then the quantity of the unknown is found from the curve.

All immunoassays require the use of labeled material in order to measure the amount of antigen or antibody present.

**A label is a molecule that will react as part of the assay, and in doing so produce a signal that can be measured in the blood: reagent solution.**

**Examples of label include**:

A radioactive compound, an enzyme that causes a change of color in a solution, a substance that produces light.

Labeled immunoassays are designed for antigens and antibodies that may be

small in size or present in very low concentrations.

The presence of such antigens or antibody is determined indirectly by using a labeled reactant to detect whether or not specific binding has taken place.

 **( Rinsho et al., 1995)**

**IMMUNOASSAY METHODS APPLIED IN PHARMACEUTICAL SCIENCE:**

1. **Radioimmunoassay**
2. **Enzyme Immunoassay**
3. **Fluoroimmunoassay**
4. **Chemiluminescence Immunoassay**
5. **Liposome Immunoassay**

**( Rinsho et al., 1995)**

**RADIOIMMUNOASSAY (RIA)**

**DEFINITION:**

* **Radio=** Use of radioactive material
* **Immuno=** Antigen antibody binding theory
* **Assay=** Detection of compound

**A binding assay in which the binder is an antibody which uses radioactivity to measure the amount of bound and/or free antigen.**

Radioactively labeled antigen is called "tracer". Radioactive isotopes are usually 3H (beta) or 125I (gamma), which emits radiation that can be measured with a beta or gamma counter.

**“OR”**

**Radioimmunoassay (RIA) also known as Radio tracer technique is an in vitro diagnosis technique using radio isotopes that measures the presence of an antigen ( e.g., hormone levels in the blood) with remarkable sensitivity and a high degree of specificity.**

**HISTORY:**

RIA could be considered a serendipitous discovery in that it arose as fallout from investigations into what would appear to be an unrelated study. To test the hypothesis that maturity onset diabetes might be a consequence of abnormally rapid degradation of insulin by an enzyme, insulinase, radioiodine-labeled insulin was administered as a tracer to study the distribution and turnover of insulin in diabetic and non diabetic subjects. It was observed that the labeled insulin disappeared more slowly from the plasma of subjects with a history of insulin treatment than from the plasma of diabetic or non diabetic subjects who had never received animal insulin. It was demonstrated that the slower rate of removal was a consequence of binding of insulin to an acquired antibody. Almost immediately it was appreciated that methodology used to study kinetics of reaction of insulin with insulin binding antibody and to determine binding capacity of that antibody could be applied reciprocally to determine the concentration of insulin in body fluid. Berson and Yalow were first to describe the word immunoassay in 1957, but it was not until several years(Berson and Yalow, 1959) later that the assay had sufficient sensitivity to measure the concentrations of insulin in circulations of humans. Dr. Rosalyn Yalow became the first female to win a Nobel Prize with her work on radioimmunoassay.

During the first decade after its discovery RIA was used primarily to measure the concentration of peptide hormones. Since these substances are present in plasma in the unstimulated state at concentrations as low as 10-12- 10-10 M, the sensitivity and specificity of RIA were required to study the dynamic interactions between the peptide hormones and the substrates which they regulate and by which they are in turn regulated. By the 1970s RIA methodology had spread from endocrinology, its first home, into many other areas of medicine, including pharmacology and toxicology, infectious diseases, oncology and hematology.

**(Yalow 1987)**

**TYPES OF RADIOIMMUNOASSAY:**

There are two types of RIA,

 1. Competitive RIA

 2. Immunoradiometric (sandwich) assays.

**Competitive assays** use radiolabeled antigen. The labeled antigen "competes" with non-radioactive antigen in the sample for a limited number of binding sites on the reagent antibody. Following incubation, the free radiolabeled antigens are removed by decanting or washing and the radioactivity of the antibody-bound antigens is measured. The radioactivity of the antibody-antigen complexes is inversely proportional to antigen concentration.

 In **Immunoradiometric (IRMA)** or sandwich assay, two antibodies are used and one is radiolabeled. In the test system, the sample is incubated with a specific antibody usually attached to a solid phase such as a plastic bead or the wall of a plastic test tube. After washing to remove unbound sample components, a radioactively labeled antibody is added. The second antibody may be directed against a different part of the antigen molecule, or it may be directed against the first antibody (e.g. anti-human immunoglobulin). The second antibody binds to the immune complexes making an antibody-antigen-antibody "sandwich." After washing to remove the unbound radiolabeled antibody, the radioactivity is measured. The amount of radioactivity is directly proportional to antigen concentration.

(**Robert et al., 2002)**

**PURPOSE OF RIA:**

It is a Radio-analytical technique that combines the specificity of an antigen-antibody reaction with sensitivity of radioactivity measurements.

This is a technique **used for** detection of micro quantities of protein, viral antigens, antibodies, hormones, steroids, tumor markers, structural proteins, vitamins, drugs and their metabolites. It can also be used for detection of pictogram quantities (10 −12 g) of biological constituents present in biological fluid.

Any biological substance for which a specific antibody exists can be measured, even in minute concentrations using this technique.

RIA has been the first immunoassay technique developed to analyze nanomolar and picomolar concentrations of hormones in biological fluids.

**RIA is used in place of bioassay in various branches of science like Biochemistry, Microbiology, and Hematology and Clinical pharmacology.**

**(Yalow 1987)**

**PRINCIPLE OF RIA:**

**RIA works on basic principle of biochemistry i.e. competitive binding between antigens for same antibody binding site.**

The competition of an analyte with its radioisotopically labeled counterpart for a limited amount of antibody is the underlying principle of this technique.

**Ag + Ag\* + Ab 🡪 AgAb + Ag\*Ab + Ag**

**Increasing the analyte concentration inhibits the binding of the labeled analyte to the antibody. This can be explained in the following manner:**

* A known amount of radioactivity labeled antigen is reacted with a limited amount of antibody, producing a solution containing antibody bound labeled antigen as well as some unbound labeled antigen. After separating antibody bound antigen from free antigen, amount of radioactivity bound to antibody is determined.
* Next same amount of labeled antigen is premixed with unlabeled antigen. Mixture is reacted with same amount of antibody as before and antibody bound antigen is separated from unbound antigen. Because unlabeled antigen competes with labeled antigen for antibody, less labeled antigen is bound to antibody.
* Greater the amount of unlabeled antigen present in reaction mixture, smaller the ratio of antibody bound radiolabeled antigen to free radiolabeled antigen.
* The B/F ratio is dependent upon the amount of nonradioactive antigen. Antigen concentration in unknown samples is determined by comparing the B/F ratio to the B/F ratios obtained by incubating varying amounts of known nonradioactive antigen with the same amount of antibody as in the unknown sample under similar assay conditions.

**The amount of Ab per tube is kept constant, the amount of antigen added (known or unknown) is the variable parameter. The added antigen will be distributed between a bound (B) and a free (F) fraction. This distribution is governed by the association constant (KA) of the Ab:**





* A graph is then plotted between ratio of antibody bound antigen / free antigen and concentration of unlabeled antigen. Antigen concentration in the sample can be read directly from the standard curve.



**1. – Ratio in unknown**

**2. - Antigen in unknown**

**Red line – binding line**

**Green line –** **free labeled antigen**

* From graph we can also calculate %F (fraction of free labeled antigen) and %B (fraction of bound labeled antigen).

(**Bhattacharya and Sinha 2006)**

 

**F = amount of free labeled antigen**

**B = amount of bound labeled antigen**

 **(Yalow 1987).**

**Flow Chart of Technique:**

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**REQUIREMENTS FOR THE DEVELOPMENT OF RIA:**

For detection of an analyte, the following are usually a part of the assay:

* + 1. Labeled and unlabeled antigens
		2. Specific antibody
		3. Standards or calibrators
		4. A method to separate the bound from free components
		5. A method for detection of the label.

**METHODS INVOLVED IN RIA:**

1. Radio labeling of the Antigen or radio labeled production
2. Preparation & characterization of the Antigen [Ligand to be analyzed]
3. Preparation of the Specific Antibody
4. Development of Assay System or separation techniques
5. **Preparation and radio labeling of antigen:**

**Antigen preparation:**

1. Synthesis of the molecule
2. Isolation from natural sources

**Radio labeling (Tagging procedure):**

The antibody-antigen interaction is characterized by a high bond energy, which ensures the formation of antibody-antigen complex, even when the two constituents are present at very low concentrations. This strong affinity of the antibody for the antigen is exploited in immunological assays in order to obtain high sensitivity. In order to measure the very low concentrations of these complexes, it is necessary to introduce in to the assay a labeled molecule, which provides a detectable signal at these concentration levels. The labeling can be of the antibody or of the antigen, depending on the type of assay used. The properties of this labeled molecule (i.e., tracer) greatly influence the characteristics of the assay and in particular its sensitivity.

The preparation of tracer consists in using any physicochemical method to couple an antigen or an antibody with an atom or molecule capable of emitting a measurable signal. The structure of the tracer must be based on that of the immunogen which served to prepare the antibodies, so as to reproduce as closely as possible the structural design implicated in recognition by the antibody sites.

 (**Grassi et al., 1987)**

**Two most commonly used radio labels in RIA** are 3 H and 125I, although 75Se, 32P, 57Co, 14 C and 131I have also been used but these have some limitations that are:

**32 P and 57 Co** = limited by stereo chemical aspects because drug naturally contain phosphorous and cobalt

**14 C** = low specific activity field

**131 I** = Short decay half life and radio degradation character on the other hand

**3 H and 125I** **have advantages as follows:**

**3 H** = Direct incorporate into molecular structure, half lives (12 years)

**125 I**= High activity and ease of counting (half lives 60 days)

 (**Haarburger 2008)**

**Technique of labeling of drug or antigen with 3 H and 125I:**

**Labeling of drug with 3H:**

Specific tritium labels are generally obtained by reducing an appropriate precursor in presence of 3H.

**Labeling of drug with 125I:**

The radioactive isotope iodine is covalently linked to tyrosine residues present on antibodies and most antigens with chloramines T or Lactoperoxidase method. Usually, 125I will take the place of a hydrogen atom on the ring of tyrosine.

**Comparison of 3H and 125I is as follow:**

|  |  |  |
| --- | --- | --- |
| **Sr. no.** | **Tritium (3H)** | **Iodine (125I)** |
| **1** | It is more efficient when relatively small numbers of samples are assayed. | It is more efficient as the numbers of samples are increased. |
| **2** | Such type of problem does not occur. | It’s having quality control problems such as damage during the reaction and radiation damage following synthesis. |
| **3** | It has advantages of long half life, higher affinity. | Its shorter half life dictates frequent preparation. |
| **4** | Purchased commercially | Produced in research lab itself |
| **5** | **Beta-radiation** is weak and therefore more difficult to measure, thus practically cumbersome.Beta rays have low penetrating power.The radioactive sample needs to be mixed with a scintillator fluid; the produced light is measured by use of a photo- multiplier ("beta-counter"). | **Gamma-radiation** has high penetrating power, is therefore easy to measure, thus practical to use. |
| **6** | The tracer immunologically behaves exactly as the cold hormone (unlabeled antigen). | The tracer immunologically does not always behave exactly as the cold hormone, due to iodination damage. |

 **(Chervu et al., 1975)**

1. **Preparation of drug-protein conjugate(ligand-antigen) to be analyzed:**

In most cases the drugs (analyzed antigen) are bind with suitable carrier protein to make conjugated antigen immunogenic. There are several reactive groups on protein carrier which can be used for the purpose of conjugation of standard drug (antigen).

These groups include the terminal amino and carboxyl groups, amino group of lysine, the carboxyl group of aspartic and glutamic acid, the phenolic group of tyrosine.

1. **Preparation & characterization of the Specific and high affinity Antibodies:**

**Preparation:**

The production of antibodies is an important process in the use of immunoassays. Antibodies can be called monoclonal or polyclonal, depending upon the technique used to produce them.

**Polyclonal antibodies**may be produced in mammals such as rabbits or sheep.

When a foreign substance enters the body, it stimulates the immune system to produce antibodies to the substance. Using this natural reaction, an analyte in as pure form as possible is injected into the animal stimulating the production of antibodies. It is directly used in assay and should be stored at 4°c. Antiserum usually contains a mixture of antibodies that recognize and bind to the same antigen, but they may attach to different epitopes.



**Monoclonal antibodies** production result in very specific antibodies that bind only to one antigen epitope. (**Brune et al., 1987)**





**Characterization:**

Characterization of antiserum is done by fractionation, immunoadsorption or immunosaturation technique.

1. **Development of suitable separation techniques to separate free from bound standard drug:**

In most assays, once the reaction between antigen and antibody has taken place, there must a way of separating reacted from unreacted analyte. This can be accomplished by different means.

**Several methods that employ physiochemical and immunological separation have been devised as follows:**

**a. Physical methods:**

Filtration, Chromatography, Electrophoresis, charcoal- dextran adsorption and ion exchange resin.

* **Adsorption on particles such as dextran-coated charcoal**:

These adsorb out the smaller unbound molecules, which are then separated from bound molecules by centrifugation or filtration. The amount of label remaining in the supernatant provides an indirect measure of analyte present in the patient's sample.

* **Separating antibody-bound from free fractions by column affinity chromatography:**

 In this method polypropylene columns containing about 150 mg of immunosorbent (goat anti-rabbit gamma-globulins covalently linked to Sepharose CL-4B) are used. Standards or unknowns, tracer and antiserum, pipetted into bottom-capped columns, are kept separated from the immunosorbent bed by a porous polyethylene disc and allowed to react for 15 min at room temperature. The reaction mixture is then allowed to pass through the columns by removing the bottom caps. Free antigen is eluted by washing the column, and discarded; antibody-bound fractions remain bound to the immunosorbent. The radioactivity in the columns is counted.

**Disadvantages:**

These tend to be time dependent and harsh so they may remove bound drug from antibody during separation.

**b. Chemical methods:**

Another means of separation involves precipitation of antigen-antibody complexes using organic solvents such as ethanol, dioxane and polyethylene glycol (PEG), and salts such as sodium, zinc, and ammonium sulfate. e.g.,

* **Using ammonium sulfate:**

Separation of complexes of antibody bound antigen from free antigen is based on fact that immunoglobulin becomes insoluble and precipitate in a solution containing 33% saturated ammonium sulfate and thus antibody bound antigen precipitate, leaving the free antigen in solution.

**Disadvantages:**

Chemical precipitation may precipitate free as well as bound drug during separation depending on physicochemical nature of drug.

**c. Solid-phase stage for separation:**

Currently, most immunoassays use a solid-phase stage for separation. Numerous substances, such as polystyrene test tubes, microtitre plates are used for this purpose.

**Procedure:**

Add known amounts of the test sample and labeled antigen into the microtitre wells. Incubate and allow the reaction to reach completion. Antigen or antibody is attached by physical adsorption and when specific binding takes place, complexes remain attached to the solid phase. Decant & wash contents of the well. This removes all unbound antigens. Radioactivity remaining in the microtitre wells is measured by a counter. **Gamma counter** is used for gamma energy emitting isotopes e.g., 125I. **Scintillation counter** is used for beta energy emitting isotopes e.g., Tritium 3H and 14 C isotopes.

**d. Second antibody method:**

It is most important physiologic procedure to precipitate bound antigen. Precipitate the antigen-antibody complexes by adding a "second" antibody directed against the first.

**Procedure:**

It is based on the fact that labeled or unlabeled antigen that is bound to immunoglobulin will be precipitated along with immunoglobulin following addition of anti immunoglobulin antibodies. As a result only unbound antigen will remain in supernatant.

RIA commonly employs rabbit antibodies to desired antigen. These rabbit antibody antigen complexes may be precipitated by addition of goat antibodies raised against rabbit immunoglobulin.

**Disadvantages:** This technique have required prolong incubation time.

 (**Paul 2013) (Chan et al., 1987)**

**ADVANTAGES OF RIA:**

Radioimmunoassay is widely-used because of its great sensitivity and specificity.

**High specificity:** The greater the specificity of the antiserum, the greater the specificity of the assay.

**High sensitivity:** Immune reactions are sensitive. Using antibodies of high affinity, it is possible to detect a few pictograms (10−12 g) of antigen in the tube.

**Accurate and precise:** This technique has gained importance because of its accuracy and precision. **(Khan et al., 2010)**

**DISADVANTAGES OF RIA:**

* Radiation hazards because it uses radiolabelled reagents
* Requires specially trained persons
* Labs require special license to handle radioactive material
* Requires special arrangements for
	+ Requisition,
	+ Storage of radioactive material radioactive
	+ Waste disposal.
* Requires expensive instrumentation for the counting of radioactivity
* Requires special attention for handling of the reagents

 (**Naik 2012) (Stevens 2009)**

**APPLICATIONS OF RIA:**

1. **RIA has become a major tool in the clinical laboratory where it is used to assay:**
* Plasma levels of:
	+ Most of our hormones;
	+ Digitoxin or digoxin in patients receiving these drugs;

**(**[**Evered**](http://www.ncbi.nlm.nih.gov/pubmed/?term=Evered%20DC%5Bauth%5D) **et al., 1970)**

* + Certain abused drugs.
* Presence of hepatitis B surface antigen (HBsAg) in donated blood.

**(**[**Katchaki**](http://www.ncbi.nlm.nih.gov/pubmed/?term=Katchaki%20JN%5Bauth%5D) **et al., 1978)**

* Anti-DNA antibodies in systemic lupus erythematosus (SLE).

**(Mackworth-Young et al., 1986)**

1. **Screening donated blood for evidence of viral contamination by:**
* HIV-1 and HIV-2 (presence of anti-HIV antibodies)

**(**[**Hodinka**](http://www.ncbi.nlm.nih.gov/pubmed/?term=Hodinka%20RL%5Bauth%5D) **et al., 1998)**

* Hepatitis C (presence of antibodies)
* Hepatitis B (testing for both antibodies and a viral antigen)

**(Gerlich 2013)**

1. **Measuring hormone levels:**
* HCG (as a test for pregnancy) **(**[**Majali**](http://link.springer.com/search?facet-author=%22Ms.+M.+A.+Majali%22) **Ms et al., 1981)**
* LH (Luteinizing hormone, determining the time of ovulation) **(Schalch et al., 1968)**
* TSH, T3 and T4 (for thyroid function) **(Burman et al., 1977)**
* Testosterone **(Federico et al., 2011)**
* Vitamin B12 ([**O'Sullivan**](http://www.ncbi.nlm.nih.gov/pubmed/?term=O'Sullivan%20JJ%5Bauth%5D) **et al., 1992)**
* Prostaglandins **(**[**Jaffe**](http://www.ncbi.nlm.nih.gov/pubmed/?term=Jaffe%20BM%5Bauth%5D) **et al., 1973)**
* Hormones (e.g., anabolic steroids, HGH) that may have been used illicitly by athletes. **(Brooks et al., 1975)**
1. **Detecting infections:**
* Sexually-transmitted agents like HIV, Syphilis, and Chlamydia

**(**[**Hodinka**](http://www.ncbi.nlm.nih.gov/pubmed/?term=Hodinka%20RL%5Bauth%5D) **et al., 1998)**

* Hepatitis B and C **(**[**Archer**](http://www.ncbi.nlm.nih.gov/pubmed/?term=Archer%20AC%5Bauth%5D) **et al., 1983)**
* Toxoplasma gondii (**Gehle et al., 1976)**
1. **Detecting allergens in food and house dust :**

**RAST:** The radioallergosorbent test to detect specific IgE antibodies to suspected or known allergens. IgE is the antibody associated with type I allergic response.

**(**[**Sampson**](http://www.sciencedirect.com/science/article/pii/0091674984900836) **et al., 1984)**



1. **Detecting illicit drugs:**
* Cocaine
* Opiates
* Δ-9-tetrahydrocannabinol, the active ingredient in marijuana

**(**[**Ledgerwood**](http://www.ncbi.nlm.nih.gov/pubmed/?term=Ledgerwood%20DM%5Bauth%5D) **et al., 2008)**

1. **Measuring "rheumatoid factors" and other auto antibodies in autoimmune diseases.**

([**Holborow**](http://link.springer.com/search?facet-author=%22E.+J.+Holborow%22) **et al., 1981)**

1. **Therapeutic drug monitoring:**
	* + Cyclosporine **(** [**Mraz**](http://link.springer.com/search?facet-author=%22W.+Mraz%22) **et al., 1988)**
		+ Digoxin. **(** [**Evered**](http://www.ncbi.nlm.nih.gov/pubmed/?term=Evered%20DC%5Bauth%5D) **et al., 1970)**

 **9. Tumour markers:**

RIA of tumour markers such as

* Alpha-fetoprotein (AFP) **(** [**Jiang XM**](http://www.ncbi.nlm.nih.gov/pubmed?term=Jiang%20XM%5BAuthor%5D&cauthor=true&cauthor_uid=12816714) **et al., 2003)**
* Carcinoembrionic antigen (CEA),
* b-HCG **for** **choroid-carcinoma**,
* Prostate specific antigen (PSA) **for prostate cancer**, are available for detection and management of cancer.

**NON CLINICAL APPLICATIONS:**

* Veterinary science
* Food processing industry
* Drug industry
* Forensic science
* Environmental monitoring.

**(Skrzipczyk et al)**

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