31 SIZE EXCLUSION CHROMATOGRAPHY

CONTAINS :

31.1. INTRODUCTION

The **size-exclusion chromatography** (or **gel-chromatography**) is a means of separation which is exclusively dependent on the exchange of solute molecules between the solvent of the mobile-phase and the same solvent within the pores of the column-packing material. In reality, it is the pore-size-range of the packing material that solely determines the molecular-size-range within which a particular separation can take place effectively.

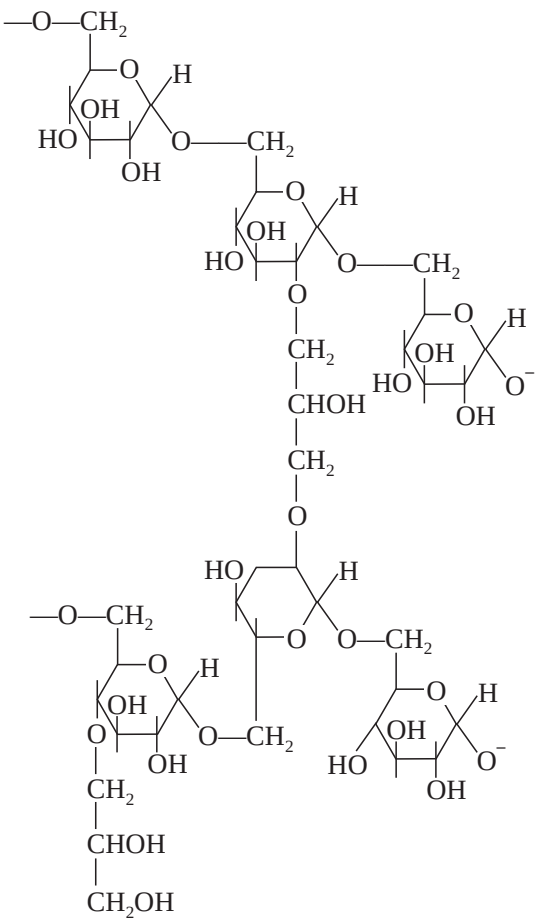
The timely adoption of the *cross-linked dextran gels* (*i.e.*, **Sephadex**) in late-fifties as a packing material for column chromatography opened an altogether new horizon of chromatographic separation whereby substances, in general, undergo separation more or less as per their molecular size.

In actual practice, the inert gels of dextran (I)-a polyglucose or other types of polymers, for instance : agarose and polyacrylamides, wherein the macromolecules invariably are cross-linked to afford a reasonably porous 3D-structure*, served as the stationary phases in size-exclusion chromatography.

The salient features of 'gels' are enumerated below :

(*i*) The extent or degree of cross-linking and obviously the sizes of the pores within the body of the gels are rigidly monitored and controlled during the course of manufacture,

* Three-dimensional (3D) structure.



(I)

(*ii*) Mostly the gels are hydrophilic in nature and evidently they swell-up in contact with water,

(*iii*) Gels having a large degree of cross-linking and a relatively large pore size usually need a larger volume of water in order to fill up the pores available within the gel-structure in comparison to the tightly linked gels, as could be seen in Table 31.1.

Table 31.1 : Characteristics of Dextran Polymer Gels

Sephadex Grade	Water Regain (g/g)	Mol.Wt.	Bed* Vol. (ml g^{-1})	Swelling Time (*h*)
Sephadex-15	1.5	1,500	2.5-3.6	3
Sephadex-75	7.5	50,000	13	24
Sephadex-200	20	200,000	30	72

(*iv*) Buffered aqueous solutions normally serve as mobile phases in size-exclusion chromatography. However, highly modified gel polymers are also available commercially (*e.g.,* Sephadex-LX) that exclusively make use of organic solvents.

31.2. THEORY

The efficiency and ability of a gel to slow down the movement of various substances downwards in a packed column with the respective gel entirely depends on the molecular size of the substance *vis-a-vis* to the

* Dry polymer basis.

pore sizes prevailing within the gel matrix. Evidently, a substance with high molecular weight is unable to diffuse into the pores of the gel and thereby moves down the column more rapidly through the channels between the grains of the gel. On the contrary, a substance having molecular size distinctly smaller than the largest pores of the gel shall naturally penetrate the pores and move with a slower pace down the column. In this manner the substances having molecular size greater than the pores shall undergo exclusion thereby affecting their elution from the column into the space immediately ahead of the relatively small molecular weight components. In other words, the substances are found to be eluted from the column strictly in order of the decreasing molecular size.

The liquid phase which is absorbed by the synthetic polymer granules (*e.g.*, Sephadex) is mostly available in a wide range as solvent for solute molecules in contact with the gel. It has been observed that the actual distribution of the solute in between the inside and outside of the respective gel granules is nothing but a criterion of the available space. However, the underlying distribution coefficient occurring between the granular and interstitial aqueous phases is found to be independent of *three* major factors, namely :

(*a*) pH

(*b*) Ionic strength, and

(*c*) Concentration of the solvent.

31.2.1. DISTRIBUTION COEFFICIENT (K_D)

The distribution coefficient is defined by the underlying expression :

$$K_D = \frac{V_R - V_O}{V_T - V_O}$$

where, V_R = Retention volume for the component of interest,

V_O = Retention volume for a non-retained component (or exclusion volume), and

V_T = Retention volume for a component that has full access to all the pores of the support (or total permeation volume).

31.2.2. PERFORMANCE

The column performance may be determined from the number of theoretical plates per metre (*n*), calculated by the help of the following expression :

$$n = \frac{5.54 V_R^2}{L W_h^2}$$

where, V_R = Retention volume* for the component of interest,

W_h = Width of the peak of interest at half peak height, measured in the same units as V_R.

31.2.3. MATERIALS

There are usually three types of materials that are employed in the size-exclusion chromatography for pharmaceutical substances which have been discussed briefly as under :

(*a*) **Agarose FC**

Presentation : Swollen beads 60 to 140 μm in diameter, available as a 4% suspension in water,

Applications : (1) It is used for the separation of proteins having molecular weights ranging from 6×10^4 to 2×10^7, and

(2) It is employed for the separation of polysaccharides having molecular weights varying from 3×10^3 to 5×10^6.

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* Retention Volume-is the distance along the baseline between the point of injection and a perpendicular dropped from the maximum of the peak of interest.

(*b*) **Agarose FC, Cross-linked**

Presentation : Prepared from agarose by reaction with 2, 3-dibromopropan-1-ol in strongly alkaline enviroment. It occurs as swollen beads 60 to 140 μm in diameter and is available as a 4% suspension in water.

Applications : (1) It is employed for the separation of proteins having molecular weights ranging between 6×10^4 to 20×10^6, and

(2) It is used for the separation of polysaccharides having molecular weights varying between 3×10^3 to 5×10^6.

(*c*) **Silica Gel FC**

Presentation : It is very finely divided power having an average particle size 10 μm with a very hydrophilic surface. It has an average pore diameter of about 30 nm. It is fairly compatible with aqueous solutions of pH 2 to 8 and also with various organic solvents.

Applications : It is employed for the separation of proteins having molecular weights ranging from 1×10^3 to 3×10^5.

31.3. APPARATUS

The apparatus for '**size-exclusion chromatography**' essentially comprises of a chromatographic column generally made up of glass having a diameter to height ratio of between 1 : 10 and 1 : 20, packed with an appropriate separation material (*e.g.,* different grades of **Sephadex**) which is capable of fractionation in the suitable range of molecular size and may be adequately temperature controlled. It is an usual practice to allow the mobile phase to pass through the column at a constant rate either by the aid of a suitably pump or simply by gravity.

31.3.1. APPLICATION OF SAMPLE

The sample is normally applied to the column by adopting one of the *five* following methods, namely :

(*i*) Directly to the drained-bed-surface with permitting the bed to dry,

(*ii*) Layered beneath the mobile-phase, provided the sample is denser than the mobile-phase,

(*iii*) Using a flow adaptor,

(*iv*) Using a syringe through a septum, and

(*v*) Using an injection valve.

31.3.2. DETECTION AND RECORDING

The outlet from the column is connected to a '**detector**' usually fitted with an **'automatic recorder'** that permits exclusively the monitoring of the relative concentrations of the various constituents present in the sample. However, one may also make use of an **automatic fraction collector** duly attached to the outlet from the column, if required. The various experimental parameters stated below are normally given in the *official monograph*, namely :

(*a*) Temperature of the column, if other than ambient,

(*b*) Nature of the packing material*,

(*c*) Composition of the mobile-phase,

(*d*) Flow rate of the mobile phase,

(*e*) Means of detection** of the sample components.

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* It must be treated, and the column packed, as per the manufacturer's instructions.

** UV-Specrophotometry.

31.4. APPLICATIONS OF SIZE EXCLUSION CHROMATOGRAPHY IN PHARMACEUTICAL ANALYSIS

The size-exclusion-chromatography may be used for *two* specific purposes in the analysis of pharmaceutical substances, such as :

(*i*) Determination of relative component composition, and

(*ii*) Determination of molecular weight.

31.4.1. DETERMINATION OF RELATIVE COMPONENT COMPOSITION

The assay method along with specific experimental parameters are duly stated in the *official monograph*. Here, *two* situations arise, namely :

(*a*) **Equivalent Responses :** In case, all of the components of the sample exhibit equivalent responses to the detector, then the relative quantity of each component may be determined conveniently by dividing each peak area by the sum of the peak areas of the components of interest, and

(*b*) **Non-equivalent Responses :** In case, the responses achieved are not equivalent, calculate the relative component composition either from the calibration curve obtained with the calibration standards specified in the official monograph or by any other method stated in the official monograph.

31.4.2. DETERMINATION OF MOLECULAR WEIGHT

The following steps may be followed in a sequential manner to determine the molecular weight of a pharmaceutical substance :

(*i*) Follow the method on the sample by employing the specified procedure laid down in the official monograph,

(*ii*) Plot a graph of the retention volume of the calibration standards as a function of the logarithm of the molecular weight,

(*iii*) The curve, thus obtained, normally approximates to a straight line within the exclusion and total permeation limits,

(*iv*) The molecular weight of the component of interest may be determined from the calibration curve, and

(*vi*) The calibration is valid only for the particular system employed under the specified experimental parameters.

The techniques of **size-exclusion chromatography** has been used effectively in checking the purity of the following pharmaceutical substances for their respective impurities, such as :

(*i*) Corticotrophin : For impurities of higher molecular weight,

(*ii*) Insulin : For proteins of higher molecular weight,

(*iii*) Human Insulin : For proteins of higher molecular weight, and

(*iv*) Plasma Protein Solution : For polymers and aggregates.

31.4.3. CORTICOTROPHIN : FOR IMPURITIES OF HIGHER MOLECULAR WEIGHTS

Materials Required : Corticotrophin : 1 mg ; acetic acid (1 M) [prepared by dissolving 57 ml of glacial acetic acid in 1000 ml of DW] : 100 ml ; sodium dodecyl sulphate (1% w/v) : 10 ml ;

Procedure : Dissolve accurately weighed 1 mg of corticotophin in 1 ml of 1 M acetic acid containing 1% w/v of sodium dodecyl sulphate. Heat the solution at 100 °C for 10 minutes and allow to cool.

The chromatographic procedure may be performed using (*a*) a column (about 85 cm × 10 mm) packed with polyacrylamide or cross-linked dextran for chromatography having a fractionation range for peptides with

relative molecular weights of approximately 1000 to 10,000 ; (*b*) 1 M acetic acid as the mobile phase with a flow rate of 7 ml per hour, and (*c*) a detection wavelength of 276 nm. Now, connect the detector, fitted with a flow-cell suitable for liquid chromatography having a volume of not more than 1 ml, to a strip-chart recorder. Set the detector and chart recorder at a full-scale sensitivity of 0.5 absorbance unit.

Equilibrate the column with 1 M acetic acid. Apply the cold solution to the top of the column using 0.4 ml per cm^2 of column cross-sectional area. The sum of the areas of any peaks eluted before the principal peak is not greater than 5.0% of the sum of the areas of all the peaks in the chromatogram.

31.4.4. INSULIN : FOR PROTEINS OF HIGHER MOLECULAR WEIGHT

Materials Required : Solution (1) : Dissolve 10 mg of insulin in 1 ml of the mobile phase ; Solution (2) Dilute 100 µ*l* of solution (1) to 10 ml with the mobile phase ; and Solution : (3) Dissolve 10 mg of procine insulin EPCRS* of bovine insulin EPCRS, as appropriate, in 1 ml of the mobile phase.

Procedure : The chromatographic procedure may be carried out using (*a*) a column (60 cm × not less than 7.5 mm) packed with silica gel for chromatography (10 µm ; pore size about 13 nm) Water 1-125 ; Toyo Soda TSK 2000 SW ; and Zorbax GF 250 are suitable ; (*b*) as the mobile phase with a flow rate of 0.5 ml per minute a filtered and degassed solution prepared by mixing 20 volumes of glacial acetic acid and 50 volumes of water, adjusting the pH to 3.0 by the addition of a 25% v/v solution of ammonia, adding 30 volumes of acetonitrile and mixing 1 and (*c*) a detection wavelength of 276 nm.

Inject 50 µl of each solution. Adjust the sensitivity of the detector so that the height of the principal peak in the chromatogram obtained with solution (2) is 50-70% of full-scale deflection. In the chromatogram obtained with solution (1) the sum of the area of any peak eluting before the principal peak is not greater than the area of the principal peak in the chromatogram obtained with solution (2) (1.0%).

31.4.5. HUMAN INSULIN : FOR PROTEINS OF HIGHER MOLECULAR WEIGHT

Materials Required : Solution (1) : Dissolve 10 mg of human insulin in 1 ml of the mobile phase, Solution (2) : Dilute 100 µL of Solution (1) to 10 ml with the mobile-phase, and Solution (3) : Dissolve 10 mg of human insulin EPCRS in 1 ml of the mobile-phase.

Procedure : The chromatographic procedure may be performed using (*a*) a column (60 cm × not less than 7.5 mm) packed with silica gel for chromatography (10 µm ; pore size about 13 nm) Water 1-125 ; Toyo Soda TSK 2000 SW and Zorbax GF 250 are suitable, (*b*) as the mobile phase with a flow rate of 0.5 ml per minutes of a filtered and degassed solution prepared by mixing 20 volumes of glacial acetic acid and 50 volumes of water, adjusting the pH to 3.0 by the addition of a 25% v/v solution of ammonia, adding 30 volumes of acetonitrile and mixing, and (*c*) a detection wavelength of 276 nm.

Inject 50 µL of each solution. Adjust the sensitivity of the detector so that the height of the principal peak in the chromatogram obtained with solution (2) is 50 to 70% of full-scale deflection. In the chromatogram obtained with solution (1) the sum of the areas of any peaks eluting before the principal peak is not greater than the area of the principal peak in the chromatogram obtained with solution (2) (1.0%).

31.4.6. PLASMA PROTEIN SOLUTION : FOR POLYMERS AND AGGREGATES

Materials Required : Plasma protein solution : 2.0 ml ; mixed phosphate buffer pH 7.0 with azide [To 1000 ml of a solution containing 1.8% w/v of disodium hydrogen orthophosphate and 2.3% w/v of sodium chloride and sufficient of a solution containing 0.78% w/v of sodium dihydrogen orthophosphate and 2.3% w/v of sodium chloride (about 280 ml) to produce a pH of 7.0 Dissolve sufficient sodium azide in the resulting solution to give a 0.02% w/v solution] : 1000 ml ;

Procedure : The chromatographic procedure may be carried out at room temperature using (*a*) a column (1 M × 25 mm) packed with a cross-linked dextran suitable for fractionation of globular proteins in the range of molecular weights from 5,000 to 350,000 (Sephadex G-150 is suitable), (*b*) mixed phosphate buffer pH 7.0 with azide as the mobile-phase with a flow rate of about 20 ml (4 ml per square centimetre) of column cross-sectional area) per hour, and (*c*) a detection wavelength of 280 nm.

Collect the eluate in fractions of about 4 ml and combine the fractions corresponding to each peak. For each combined fraction carry out the determination of nitrogen as per BP (1993). Not more than 10% of the total nitrogen is present in the combined fraction associated with non-retained proteins.

THEORETICAL AND PRACTICAL EXERCISES

1. What is the importance of **'Size-exclusion chromotography'** (SEC) in the assay of '*drugs*' ? Explain.

2. Expatiate the theoretical aspects of SEC with specific reference to the following :

(*a*) Distribution coefficient, (*b*) Performance, and

(*c*) Materials.

3. How would you accomplish the following **'assays'** with SEC :

(*i*) Determination of relative component composition,

(*ii*) Determination of molecular weight,

(*iii*) Impurities of high-molecular weight in **'Corticotrophin'**,

(*iv*) Proteins of high molecular weight in **'Insulin'/'Human Insulin'**,

(*v*) Polymers and aggregates in **'Plasma Protein Solution'.**

RECOMMENDED READINGS

1. Beckett, AH and JB Stenlake, **'Practical Pharmaceutical Chemistry',** Part II, 4th ed., London, The Athlone Press, 1988.

2. Jeffery, GH, J. Bassett, J. Mendham and RC Denney, **'Vogel's Textbook of Chemical Analysis,** 5th ed., New York, Longman Scientific & Technical, 1989.

3. **British Pharmacopoeia,** Vol. I and II, London, HMSO, 1993.

PART VI

MISCELLANEOUS ASSAY METHODS

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32 RADIOIMMUNOASSAY

CONTAINS :

32.1. INTRODUCTION

The introduction of **radioimmunoassay** (RIA) and its subsequent development as a possible versatile tool in wide spheres of science, occurred empirically to the initiators. Radioimmunoassay was primarily developed by Berson and Yalow* (1959) for the quantitative measurement of insulin in human plasma, which eventually not only revolutionized endocrinilogy as such but also paved the way for the clinical chemistry laboratory practice in general. As on date RIA principles have found wide application in the field of drug analysis, pharmacokinetic studies, drug-therapy monitoring and above all the immunodiagnosis in medicine to mention but a few. Specifically RIA measures the actual effect of changing concentrations of a particular substance present in a biological fluid (*e.g.*, blood, plasma, urine) based on an *in vitro* system consisting of radioactive standards of the same substance and a specific antibody. In a true sense, RIA is nothing but an indirect method of analysis because it does not make use of either the radioactive standard or the antibody present in the original sample.

Before the emergence of radioimmunoassay as an acceptable analytical technique, a number of other methods were employed for the analysis of 'drugs' in the plasma. Prominent among these methods were thin-

* Berson, SA and RS Yalow, **J. Clin. Invest., 38,** 1996, 1959.

layer chromatography (TLC), **gas-liquid chromatography** (GLC), **spectrofluometry** (SPF) and ordinary **radiolabelling assay**. The above methods, undoubtedly, have certain advantages to their credit ; however, the disadvantages outnumbered the advantages, as stated below :

Disadvantages

(1) Non-specificity of the technique,

(2) Non-sensitivity of the method,

(3) Involvement of the processes of extraction, purification and concentration of the specimen under investigation,

(4) Heat treatment of the specimen resulted invariably in degradation and destruction of the substances, and

(5) Many processes involved ultimately make the analysis rigorous and unnecessarily sluggish.

On the contrary, RIA provided a specific, sensitive, rapid, convenient, reliable, reproducible and less expensive assay methods for biological fluids.

The introduction of enzyme immunoassay (EIA) and similar allied immunoassay techniques in early eighties showed, in fact, a brighter path towards quantitative analysis.

RIA technique has splendidly made available to the drug analyst, endocrinologist, physiologist, pharmacologist, clinical chemist and biochemist a very sensitive, specific and comparatively easier method for the quantitative measurement of serum or plasma drug, hormones, enzyme concentrations, besides, drug concentrations in biological fluids. It has also proved to be equally important in pharmacokinetic studies and in acute monitoring of patient drug therapy according to Mule *et al** (1974).

Skelley *et al*** (1973) listed a number of substances that may be determined quantitatively by the help of the RIA method, namely : **nucleic acids, proteins, enzymes, prostaglandins, steroidal hormones, antibodies, cancer and viral antigens, vitamins, and drugs together with their respective metabolites.**

Importantly, the pioneer work or Oliver and coworkers*** (1968) and followed by valuable and meaningful contributions by Landon and Moffat**** (1976) proved beyond any reasonable doubt the efficacy of RIA in the quantification of a host of **pharmaceutical substances**.

32.2. THEORY

The basic underlying principle of **radioimmunoassay** utilizes the reaction between an antigen• (hapten)•• and its specific antibody. Small molecules (micromolecular) for instance : drugs that may serve as haptens and can normally be made antigenic by coupling them chemically to a macromolecular substance, such as : *protein polysaccharide, carbohydrate* etc. The hapten is obtained from a non-antigenic compound (micromolecule) *e.g., morphine, cartelol etc.*, which is ultimately conjugated••• convalently to a carrier•••• macromolecule to render it antigenic.

Animals normally develop antibodies••••• to the injected immunogenic substance as part of their natural immune response. The serum derived from these animals is used as the antibody source and tested with reference

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* Mule, SJ, ML Bastos and D. Jukofsky, **Clin. Chem., 20,** 243, 1974.

** Skelley, DS, LP Brown and P.K. Besch, **Clin, Chem., 19,** 146, 1973.

*** Oliver, GC et al, **J. Clin. Invest., 47,** 1035, 1968.

**** Landon, J., and AC Moffat, **Analyst, 101,** 225, 1976.

• **Antigen :** The substance against which antibody formation was intended (in this case, the **drug**) ;

•• **Hapten : (Ligand) :** A small, molecule, such as a drug, that must be coupled to a carrier molecule to induce an immune response, but capable of combining with specific antibodies. ;

••• **Conjugate :** The combined hapten and carrier ;

•••• **Carrier :** A protein, polypeptide, or inert matrix that is coupled to the hapten to form an immunogen;

••••• **Antibody :** An immunoglobulin that will bind with an antigen (ligand or hapten) or immunogen ;

to their specificity, sensitivity or affinity at their **titer level**. By specificity, is meant the lowest concentration of a compound which can be detected in undiluted body fluid. Generally, it is referred to as the ''**detection limit**'' or the ''**cut off level**''.

Sensitivity defines the degree to which an assay can distinguish one compound from another of the same nature and an **immunoassay** is a function of the particular antibody molecules contained in the antiserum. Specificity of the antiserum is a function of the particular antigen used to immunize the animal. Affinity usually measures how strongly bound is the antigen to the antibody. **Titer** refers to the concentration level of, in the context of the usage, antibody contained in the obtained serum.

Immunological reactions by virtue of their specificity allow the discrete identification of single molecular entities in the presence of many-fold higher concentrations of either multiple or chemically identical molecular entities. However, it is pertinent to be noted here that both **immunological** and **immunochemical techniques** are capable of providing the much sought after assay systems for *pharmaceutical substances* present in complex mixtures without the necessity of undergoing through the tedious and cumbersome process of prior extraction and purification required frequently for their respective biological and chemical tests. Interestingly, the **radioimmunochemical methods** possess the additional advantages of offering exquisite sensitivity as well as enhanced specificity*.

32.2.1. HAPTEN DETERMINANTS AND PURITY : THE KEY TO IMMUNOLOGICAL SPECIFICITY

It has since been recognized as a well established phenomenon that is possible to hook-up a micromolecule (drug) to a macromolecule (protein, polypeptide, polysaccharide) to render it antigenic, inject the resulting conjugate into an immunologically competent animal and subsequently harvest antibodies which includes those bound to the hapten moiety. Nevertheless, the animal should be genetically a responder with regard to the specific macromolecule carrier and even so to the micromolecule moiety of the immunogenic conjugate. Apparently, it may appear as the most efficient and easiest means to hook-up the micromolecule being made haptenic by any of its available chemically reactive functional groups to the selected carrier molecule.

But unfortunately, no matter how many competent animals are immunized with such an immunogenic conjugate, the antisera thus generated cannot contain a population in the total antibody immunoglobulin (IgG) pool that will recognize the chemically reactive group used for coupling to the carrier portion of the conjugate moiety. In case, only a small quantum of antigenic determinants** exist in the hapten before conjugation to macromolecule the loss of even one functional group can turn out to be critical.

32.2.2. IMPORTANCE OF ANTIGENIC DETERMINANTS

These are, namely :

(*i*) The functional groups of the hapten should remain unblocked in the conjugate molecule,

(*ii*) These chemical functions are primarily responsible for metabolic activity ; besides, all active functions of a small hapten should remain accessible in the hapten carrier conjugate to obtain the most exquisitely specific antibody immunoglobulin (IgG) population of which the immune system is capable,

(*iii*) The fewer the active functions are available to serve as haptenic determination, the lesser will be the specificity of the reaction in radioimmunoassay ; in other words, the greater the number of antigenic determinants in a hapten molecule the more specific shall be its reaction with its antibody.

Example : Blockade of a single hydroxyl group of morphine in the preparation of morphine immunogen results in an antiserum that is entirely unable to distinguish **homologous morphine forms** from its **corresponding surrogates with unavailable hydroxyl(s)*****. Further, the antiserum produced by immunization with such a morphonyl immunogen reacts with codeine either equally or better than morphine.

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* Pincus, G., KV Thimann, and E.B. Astwood, **The Hormones,** New York, Academic Press, 557, 1964.

** Functional groups that determine antigenic properties.

*** Spector, S. and CW Parker, **Science, 168,** 1347, 1970.

(*iv*) All chemically reactive functions of a pure derivative, not particularly those which coincide with physiological activity, must remain undistorted and accessible to avail themselves as immunological determinants.

32.2.3. ANALYSIS BY COMPETITIVE ANTIBODY BINDING OR ISOTOPICALLY LABELLED COMPOUNDS

Radioimmunoassay is nothing but a competitive binding assay employing the principle of reversible binding of a labelled antigen to its specific antibody ; and the ability of unlabelled antigen not only to compete in the reaction but also to displace labelled antigen from antibody. Nevertheless, the antibody and labelled antigen are always present as limiting factors and the concentration of unlabelled antigen (present either as standard solution or as sample under examination) is increased continually. It has been observed that the percentage of antibody-bound labelled antigen declines progressively as a consequence of saturation of the combining sites on the antibody molecule.

The principle governing radioimmunoassay has been duly illustrated in Figure 32.1.

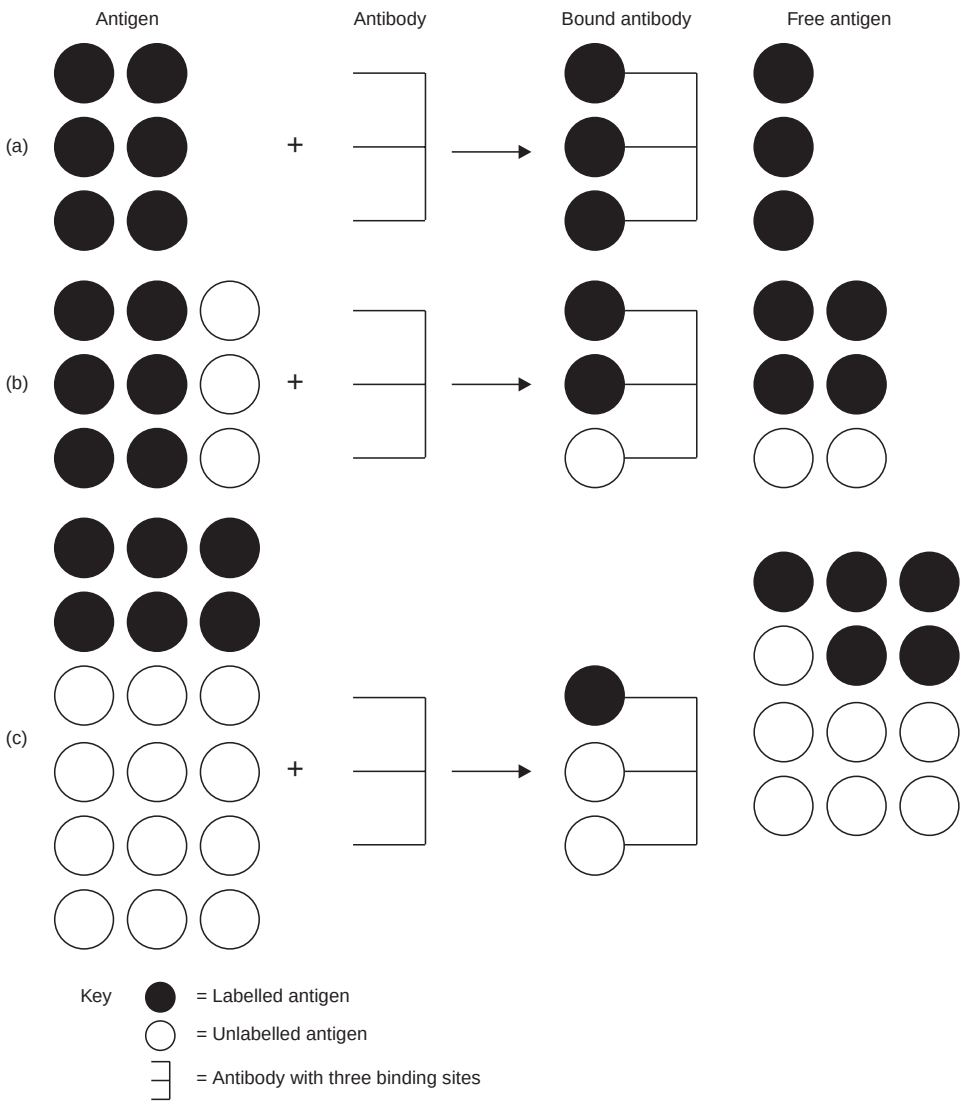


Figure 32.1 : Schematic Illustration of Competitive Binding in Ideal Radioimmunoassay.

An ideal behaviour has been assumed in Figure 32.1, whereby most radioimmunoassay very closely approach this condition. In order to fulfill the requirements of an ideal behaviour the following criteria must be accomplished, namely :

(*i*) The non-radioactive antigen (A) and radioactive antigen (A*) are indistinguishable chemically *i.e.*, both of them are identical chemically,

(*ii*) The two reactions ultimately go to completion *i.e.*, the equilibrium constants of the binding of labelled and unlabelled antigen to antibody are not only equal but also are so huge in number that they may be regarded as infinite,

(*iii*) The antigen and antibody usually react in the ratio 1 : 1, and

(*iv*) There are no cross reactions observed in the medium *i.e.*, the antibody being specific only for the single antigen indicated in the reaction or being determined.

The main objective of RIA is to determine the concentration 'C' of a non-radioactive antigen (unlabelled). Hence, in order to conduct RIA-a standard curve first to be made where 'C', concentration of non-labelled antigen in standard solution, is plotted as a function of radioactivity. It is usually accomplished by saturating the antibody binding sites with radioactive or labelled antigen, adding known concentration of the non-radioactive (hapten) antigen, in standard solution, to the reaction mixture for the unlabelled antigen from its binding site on the antibody. It is a normal practice, to measure radioactivity with each known unlabelled antigen added (concentration) which is plotted along the X-axis against the radioactivity Y-axis. This is also known as the **'close-response curve'**.

If a radioactive-labelled form of a substrate (A*) is added to a plasma containing unlabelled-substrate (A) and a limited amount of its specific binding antibody (P), then assuming a dynamic equilibrium exists between (A) and (P), (A*) shall distribute itself evenly among the unlabelled substrate (A). If the binding affinity between (A) and (P) is very high, virtually all the (A*) added will be found until (P) is saturated and at equilibrium. Thus, we have :

$$\frac{(A - P + A^* - P)}{\text{Total } (A + A^*)} \text{ or } \frac{A^* - P}{\text{Total } A^*} \text{ and } \frac{A - P}{\text{Total } A}$$

where, (A* – P) = Antibody labelled antigen-complex, and

= (A – P) = antibody unlabelled antigen-complex.

At this juncture, if further (A) is added, it will also compete for the same binding site so that (A* – P) shall be reduced. Still further additions of (A) will cause the (A* – P) concentration to be reduced further.

Under these prevailing circumstances the reduction in (A* – P) complex concentration taking place may be predicted as follows :

Assuming that P (antibody) has 200 binding sites available and at the initial stage only 20 molecules of (A) is present, sufficient (A*) is added so as to saturate P *i.e.*, 180 molecules of (A*). Therefore, virtually all are bound so that :

$$\frac{(A - P + A^* - P)}{\text{Total } (A + A^*)} \times \frac{100}{1} = 99 \text{ to } 100\%$$

If, then 100 molecules of A are added, there is a total of 300 molecules of (A* + A) competing for 200 binding sites on the antibody (P). Now, when an equilibrium is established, the percentage bound is given by the expression, :

$$\frac{(A - P + A^* - P)}{\text{Total } (A + A^*)} \times \frac{100}{1} = \frac{200}{300} \times \frac{100}{1} = \frac{A^* - P}{\text{Total } A^*} \times \frac{100}{1}$$

or

$$= \frac{120}{80} \times \frac{100}{1} = 66.6\%$$

If a further 100 molecules of A are added at this stage, the percentage bound shall become :

$$\frac{200}{400} \times \frac{100}{1} = \frac{90}{180} \times \frac{100}{1} = 50\%$$

Thus, continuing with further additions of (A), each of 100 molecules at a time will ultimately give rise to two typical RIA-Standard Curves as depicted in Figure 32.2 and Figure 32.3 respectively.

Form Figure 32.2, it is quite evident that the percentage of radioactive compound bound A* decreases with the continual addition of unlabelled compound A.

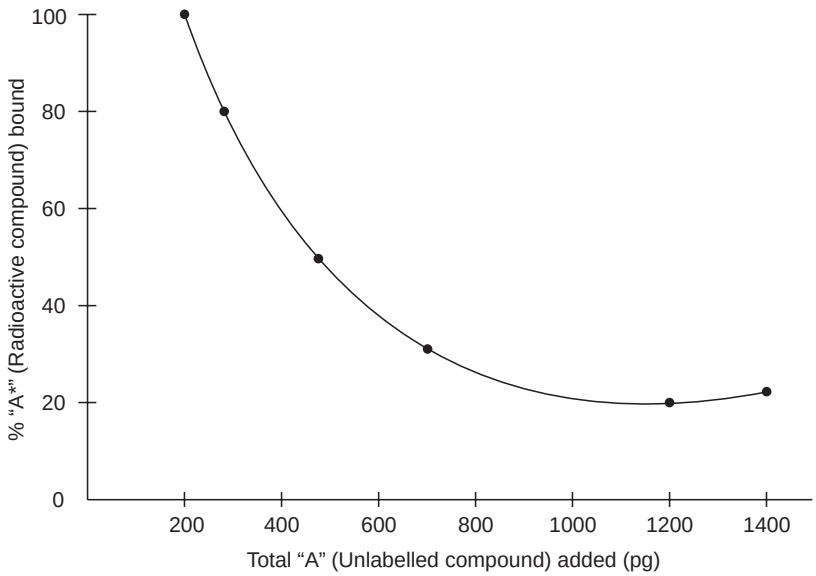


Figure 32.2 : A Typical RIA Standard Curve.

Figure 32.3, depicts the plotting of the percentage inhibition of labelled compound binding A* against the continual addition of unlabelled compound A thereby giving rise to a straight line.

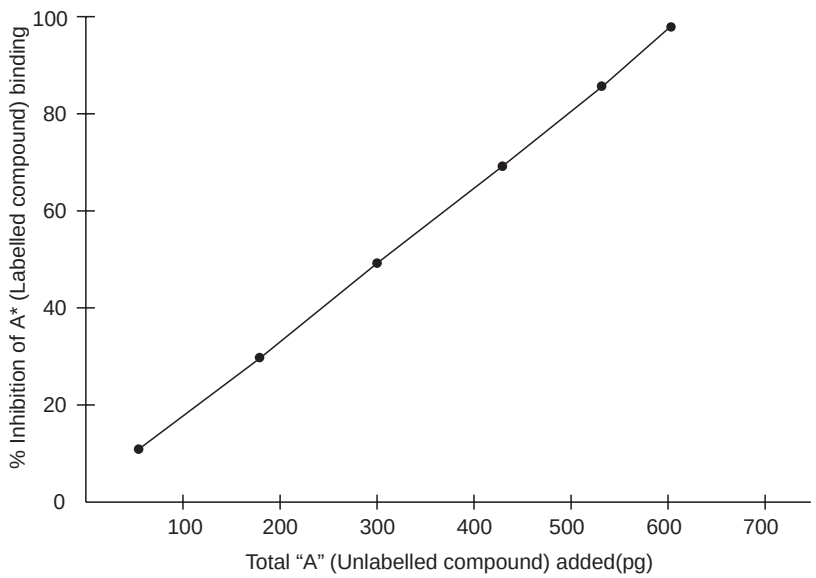


Figure 32.3 : A Typical RIA Standard Curve.

The following important points may be observed :

(*a*) In place of pure unlabelled A, a sample of plasma from which all the antibody P has been removed duly, and which contains an unknown amount of A, is added to the same system, it may be quantitated as per the respective observed fall in A* – P complex concentration that it causes ultimately,

(*b*) It is pertinent to mention here that the validity of radioimmunoassay procedure solely depends upon the identical behaviour of standards as well as unknowns (*i.e.,* unlabelled antigenic substance in unknown sample being assayed). However, this particular condition may be tested and verified by making multiple dilutions of an unknown sample and subsequently determining whether the curve of competitive inhibition of binding is superimposable on the standard curve employed for the respective assay. Failure to fulfill this condition precludes a truly quantitative estimation[+], and

(*c*) A crude hormone preparation is found to be satisfactory enough both for immunization and for use as a standard, but for the purpose of comparison of values collected from various laboratories, a generally available reference preparation must be used as a standard solution.

32.3. INSTRUMENTATION

The *two* most vital equipments essentially required for radioimmunoassay (RIA) are, namely :

(*i*) Centrifuge, and

(*ii*) Radioactive Counters.

These two equipments shall now be discussed briefly as follows :

32.3.1. CENTRIFUGE

A centrifuge which is capable of generating 1200-2500 rpm using swing-bucket-rotor or 3500 to 4000 rpm using a fixed-angle-head rotor can be employed effectively. However, the former type is preferred because of the fact that here the pellet is formed at the bottom of the test tube and the supernatant layer is more easily removed in comparison to the latter type where the pellet is formed at an angle. In case, a centrifuge having relatively less gravitational force is employed then it is absolutely necessary to enhance the centrifugation time until suitable pellets are formed duly.

32.3.2. RADIOACTIVE COUNTERS

In usual practice, *two* types of radioactive counters are mainly employed depending on the type of radioactive substance used, namely :

(*a*) Gamma Counters, and

(*b*) Scintillation Counters.

32.3.2.1. Gamma Counters

These are used invariably for the **gamma-energy emitting isotopes**, for instance : ^{125}I-the more common iodine-isotope.

32.3.2.2. Scintillation Counters

These are mostly used for counting **beta-energy-emitting isotopes**, such as : tritium ^{3}H and ^{14}C-(Carbon-14) isotopes.

First and foremost, radioimmunoassays were universally based on the ^{3}H or ^{14}C isotope labelling technique, but this has the main disadvantage of using liquid-scintillation counting. Therefore, the comparatively much simpler technique of gamma-ray counting by labelling compounds with ^{124}I, ^{125}I, or ^{131}I is now being increasingly utilized wherever such labelling is practically feasible.

+ Yellow, RS, SM Glick, J. Roth and SA Barson, Radioimmunoassay of human plasma ACTH., **J. Clin, Endocrinol., 24,** 1219, 1974.

32.4. METHODOLOGY OF THE ASSAY

The methodology of the radioimmunoassay have been studied extensively and outlined in a sequential manner as follows :

(1) Mix a fixed volume (fixed concentration) of antiserum containing the specific antibody with a constant amount of radiolabelled antigen,

(2) Incubate it for come specified duration at an appropriate temperature, usually + 4 °C,

(3) A definite volume of the sample containing the hapten to be measured is added to the reaction test-tube,

(4) The antibody reacts with both the radioactive and unlabelled hapten forming an antibody-radiolabelled antigen and antibody-unlabelled antigen complexes,

(5) Since, both the radioactive and non-radioactive antigens (haptens) are more or less chemically and immunochemically the same, they will eventually compete for the limited number of antibody sites available ; thus, the amount of radioactivity that ultimately combines with the antibody will be an inverse function of the amount of unlabelled hapten competing for these sites,

(6) The radioactivity falls because the unlabelled antigen dilutes it *i.e.*, reducing the number of labelled hapten combining with the antibody,

(7) The counts obtained from the radioactivity are used to determine the hapten concentration in the sample, the interpretation being done on the standard curve, and

(8) RIA is an exquisitely sensitive assay method that is capable of measuring with great accuracy (hapten) concentrations in nanograms and picograms utilizing very small volumes of the sample.

Note :

(*i*) *In order to measure the radioactivity in the labelled hapten-antibody complex of the free hapten* (*labelled*) *a convenient means of separating these fractions is usually adopted,*

(*ii*) *The method of assaying the radioactivity of the bound and/or unbound fraction following separation, solely depends on the nature of the isotope and on the method utilized for the separation of the bound and unbound fractions,*

(*iii*) *Thus, one may actually determine either the antibody bound fraction or the unbound fraction routinely, but in the preliminary experiments it is always necessary to determine both these fractions, and compare them with a standard containing the total number of counts added in order to make sure that there are no losses unaccounted for,*

(*iv*) *The validity of RIA entirely depends upon the identical behaviour of standard and labelled substance unknown, and not on the identity of the labelled tracer and the unknown. Hence, the experimental conditions of incubation of standards and unknowns must be identical for any factors that might affect the extent of the immunochemical reaction, pH, ionic composition, protein content or any other substances of interest. However, these conditions may be tested conveniently and can be controlled effectively by preparing standards in hormone free plasma at the same dilution at which unknowns are assayed.*

32.5. APPLICATIONS OF RADIOIMMUNOASSAY (RIA) IN PHARMACEUTICAL ANALYSIS

The scope of applicability of radioimmunoassay is rapidly expanding with the dawn of each day as RIA is being developed for newer pharmaceutical substances. It has attained wide recognition and application both *in vitro* and *in vivo* measurements of compounds of interest like insulin, gastrin, glucagon, and growth hormones on one hand ; whereas drugs like :

Morphine	—	Narcotic analgesic,
Hydromorphone and Hydrocodone	—	Narcotic analgesic, antitussive and antipyretic,

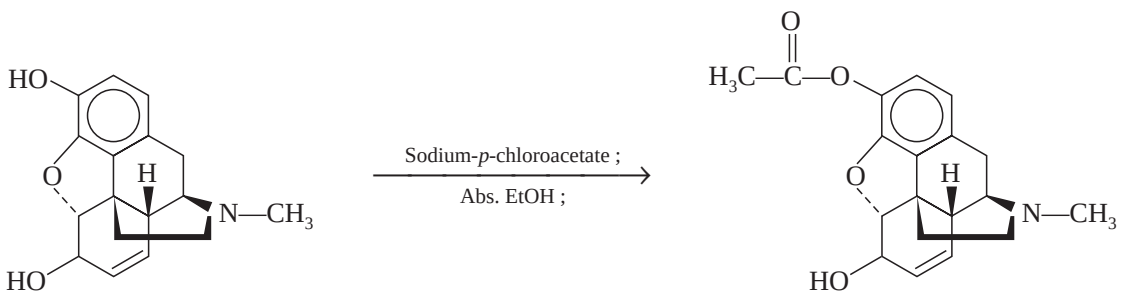
on the other hand.

Clonazepam	—	Sedative and anticonvulsant,
Flurazepam	—	Hypnotic and anticonvulsant,
Chlordiazepoxide	—	Sedative
Barbiturates	—	Hypnotic and anticonvulsant,
Flunisolide	—	A steroid having marked anti-inflammatory activity,
Neobentine	—	A novel antidysrhythmic and antifibrillatory agent,
Carteolol	—	B_1-Adrenoreceptor blocker used in hypertension and arrthmias,

RIA of some of these drugs will be discussed in the sections that follows :

32.5.1. RADIOIMMUNOASSAY OF MORPHINE

Synthesis of Immunogen : Morphine is first converted to 3-*o*-carboxymethyl-morphine by reacting the free base with sodium-*p*-chloroacetate in absolute ethanol :



Morphine
(I)

3-*o*-Carboxymethyl-morphine
(m.p. 292-293°C)
(II)

The product (II) is coupled to bovine-serum albumin by dissolving the former in distilled water containing the latter, maintaining the pH of the resulting mixture to 5.5 and 1-ethyl-3-(3-dimethyl-aminopropyl) carbidiimide was added. The mixture is incubated overnight at room temperature and then dialyzed against distilled water to cause purification. The resulting purified product carboxy-methyl-bovine-serum conjugate is then labelled with tritium.

Antiserum Production : The immunogen, carboxymethylmorphine-bovine-serum-albumin, is emulsified with equal volume of complete Freund's adjuvant*. Initial immunization doses are injected into the New Zealand albino rabbits and later on this followed up with booster injections after a period of 6 weeks. The antiserum titer is determined with each booster dose injection and is duly harvested when the titre value is maximum. This is diluted suitably and employed in the radioimmunoassay**.

RIA-Procedure : The various steps followed are as stated below, namely :

(1) Various dilutions of antiserums are incubated in the presence of fixed concentration of (^{3}H)-dihydromorphine, and after incubation, a neutral saturated ammonium sulphate solution is added to all the tubes,

(2) The complete precipitate, sedimented by centrifugation at 5000 rpm is washed twice with 50% ammonium sulphate solution,

(3) The washed-precipitate, containing antibody-bound morphine, is dissolved in NCS-solubilizer, and the radioactivity is counted with the help of a Packard-Iri-card Liquid Scintillation Spectrometer,

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* **Adjuvant—**An oily substance that will form an emulsion aqueous solutions/suspensions of immunogen to enhance the antibody response. Complete adjuvant also contains *Tubercele bacilli.*

** Spector, S. and CW Parker, **Science, 168,** 1347, 1970.

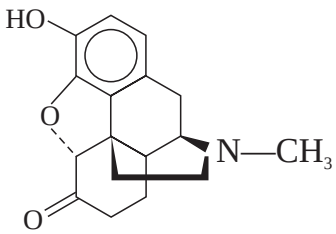
(4) The tube which contained radioactive dihydromorphine and antiserum but no unlabelled morphine, served as a measure of maximum antibody-bound radioactivity,

(5) The addition of increasing amount of unlabelled morphine to a fixed amount of (^{3}H) dihydromorphine and antiserum results a competitive inhibition of the labelled dihydromorphine for the formation of the antibody-hapten complex, and

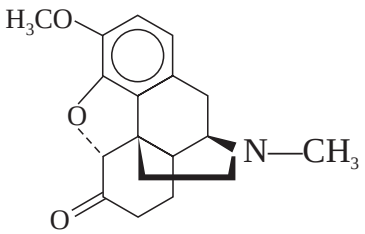
(6) The assay sensitivity limit is found to be 100 pg of unlabelled-morhine per tube that caused 20% binding inhibition of labelled-dihydromorphine, (see Figure 32.3).

32.5.2. RADIOIMMUNOASSAY OF HYDROMORPHONE AND HYDROCODONE IN HUMAN PLASMA

Hydromorphone (I) and **hydrocodone** (II) belong to the **morphine group of drugs** and are used invariably in combination with other ingredients in a number of proprietory antitussive and analgesic antipyretic mixtures. However, interest in the pharmacokinetics of hydromorphone and hydrocodone in human subjects required an adequate assay for drug levels in plasma.



Hydromorphone
(I)



Hydrocodone
(II)

RIA for hydromorphone*, ** and hydrocodone*** are fairly sensitive in the nanogram per millilitre range but essentially require the preparation of a specific antibody. The laid-out RIA method is quite capable of estimating the above drugs within a range of 2.5-20 ng ml^{-1} using standard 100 µl plasma sample only.

RAI is carried out using morphine-6-antiserum and tritiated dihydromorphine (commercially available). The free-drug is separated from bound drug using dextran coated charcoal and an aliquot of the supenate containing the antiserum-bound-drug is subsequently counted for radioactivity. However, the radioactivity measurements are normally ascertained in a Liquid Scintillation Counter provided with 20-ml glass scintillation vials.

Materials Required

(*i*) **Lyophilized morphine-6-antiserum :** It is diluted 1 : 20 with phosphate buffer prior to use,

(*ii*) **^{3}H-Dihydromorphine Solution :** It is prepared by diluting 2 µl of the radiolabelled compound in ethanol to 10 ml with phosphate buffer so that each 0.1 ml of solution contained 83 pg (0.5 mole),

(*iii*) **Dextran-coated chrocoal suspension :** It is prepared by mixing 2.5 g of charcoal in 100 ml of distilled water with 2.5 g of dextran in 100 ml of distilled water, and eliminating the fine particles by centrifugation, and

(*iv*) **Preparation of Saturated Solutions :** Individual stock solutions containing the equivalent of 200 µg of I or II base line are prepared using weighed quantities of the respective powders dissolved in distilled water. Dilutions of the drugs are made in individual 10 ml volumetric flasks to yield drug concentrations of 2.5, 5.0, 10.0 and 20 ng ml^{-1} for I and 10.0, 20.0, 40.0 and 80.0 ng ml^{-1} for II. The dilutions are made using blank plasma and phosphate buffer solutions.

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* Wainer, BH, EW Fitch, J. Friend and R.M. Rothberg, **Clin. Immunol. Immunopathol, 3,** 155, 1974.

** Findlay, WJA, EC Jones and RM Welch, **Drug Metab, Dispos., 7,** 310, 1979

*** Findlay, WJA, JT Warren, JA Hill and R.M. Welch, **J. Pharma Sci 70,** 642, 1981

RIA-Procedure : The different steps to be followed are stated below, namely :

(1) Various dilutions of unknown plasma, morphine-6-antiserum, ^{3}H-dihydromorphone are prepared afresh,

(2) The unknown plasma (0.1 ml) is incubated directly with morphine-6-antiserum (0.1 ml) and buffer (0.3 ml) for a duration of 50 minutes at room temperature (20 ± 2 °C) and immediately followed by 10 minutes at 4°C,

(3) The ice-cold dextran-coated-charcoal suspension (0.1 ml) is added to all the above tubes, followed by immediate mixing and incubation for 10 minutes at 4°C,

(4) All the tubes are then centrifuged for a period of 15 minutes at 3000 rpm,

(5) A small portion (0.2 ml) of the supernate is removed and placed in a scintillation vial containing 0.5 ml of distilled water and 5 ml of scintilation fluid,

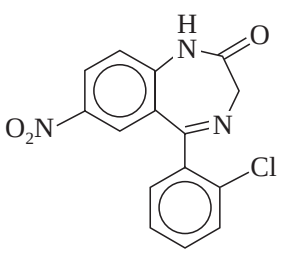
(6) The contents of the scintillation vial are mixed thoroughly, and the radioactivity is measured in a Liquid Scintillation Counter for 10 minutes,

(7) Duplicate hydromorphone 2.5, 5.0, 10.0 and 20.0 ng ml^{-1} or hydrocodone 10.0, 20.0, 40.0, and 80.0 μg ml^{-1} standards are accurately assayed concurrently and the data is plotted in a graph, and

(8) The regression equation, calculated from the standard solutions in each collection, is employed to determine quantitatively the drug concentration present in individual plasma samples.

32.5.3. RADIOIMMUNOASSAY OF CLONAZEPAM

Colonazepam belongs to the class of **1, 4-benzodiazepines** that has been found to be therapeutically effective in controlling minor motor seizures *i.e.,* petitmal epilepsy in humans*



Clonazepam

Synthesis of Immunogen : The 3-hemisuccinyloxy derivative of clonazepam is covalently coupled to bovine serum albumin employing the mixed-anhydride method suggested by Erlanger and coworkers** (1959). After successive dialysis against dioxane-water borate buffer and water, the immunogen *i.e.,* clonazepam-bovine-serum-albumin conjugate is isolated by lyophilization.

Preparation of ^{3}H-Labelled Clonazepam : ^{3}H-Clonazepam is prepared by tritium exchange employing dimethyl formamide-titrated water having a specific activity*** of 100 ci g^{-1}. The resulting product is subsequently purified by silica-gel-column-chromatography, thereby yielding a material which has a specific activity of 4.32 mci mg^{-1}. This specific method of introducing ^{3}H (tritium) probably provided exchange chiefly at C-3 position****.

Antibody Production : A thick emulsion of the immunogen (clonazepam-bovine-serum-albumin-conjugate) is prepared employing complete Freund's adjuvant and two New Zealand white female rabbits are immunized intradermally at multiple sites with the immunogen emulsion. The animals are then administered

* Kruse, R., **Epilepsia, 12,** 287, 1971

** Erlanger, BF, R. Borker, S.M. Beiser, and S. Liebermann, **J. Boil. Chem, 234,** 1090, 1959.

*** **Specific activity**-is a unit that expresses the amount of radioactivity as a function of the mass of the material.

**** Dixan, W.R., K.E. Fahrenholtz, W. Burger and C. Perry, **Res. Commun. Chem. Pathol. Pharmacol. 16,** 121, 1977.

with booster doses intravenously with immunogen emulsion at monthly intervals, and serum is harvested 10-14 days after each administration. Both rabbits produced satisfactory titers of antibodies to clonazepam within a period of three months following the initial immunization. The resulting serum is pooled, diluted suitably and employed in the radioimmunoassay.

RIA-Procedure : The various steps involved in the RIA-procedure for clonazepam are enumerated below, namely :

(1) A constant small (volume) portion of the control plasma is added to constant small (volume) portion of standard clonazepam in small test-tubes to generate a calibration (standard) curve,

(2) Appropriate controls are included by adding the control plasma to small portion of buffer solutions,

(3) Each unknown plasma sample is added to tubes containing buffer solution then the titrated (^{3}H)-colnazepam solution followed by diluted antiserum is added,

(4) The contents of each tube are mixed thoroughly and allowed to stand at room temperature for sometime,

(5) Saturated ammonium sulphate solution is added to precipitate the globulin-bound-^{3}H- clonazepam and after mixing, the tubes are allowed to stand for 15 minutes at 0 °C,

(6) The tubes are subsequently centrifuged at 3000 rpm,

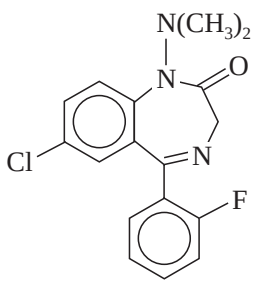
(7) Each supernate, containing the unbound ^{3}H-clonazepam, is decanted into a scintillation vial and toluene is added,

(8) The samples are assayed for ^{3}H-activity in a liquid scintillation counter, and

(9) All samples including the standards, unknowns and controls are assayed in duplicate and the average of the ^{3}H-counts is employed for the percentage of binding.

32.5.4. RADIOIMMUNOASSAY OF FLURAZEPAM IN HUMAN PLASMA

Flurazepam belongs to the class of **hypnotic agent** used for the treatment of insomnia.



Flurazepam

Synthesis of Immunogen (Hapten) : 3-Hydroxy flurazepam is refluxed with succinic anhydride in dichloromethane containing triethylamine to produce the desired hapten, 3-hemisuccinyloxy-flurazepam. It is coupled covalently to bovine-serum-albumin by the mixed-anhydride procedure developed by Erlanger *et al* (1959). The resulting conjugate is purified by dialysis against sodium bicarbonate solution followed by dialysis against distilled water and finally isolated by lyophilization.

Immunization and Antibody Production : The immunogen 3-hemisuccinyloxyflurazepam, is emulsified with complete Freund's adjuvant. It is injected intradermally into two female New Zealand albino (white) rabbits. Repeated doses are administered twice at interval of two weeks. Subsequently, booster injections of the thick-immunogen-emulsion-paste are administered after a span of 6-weeks. The antibody is harvested when its titer level is high enough, diluted to the suitable-level and employed in the RIA.

RIA-Procedure : The different steps followed in the RIA-procedure are as given below :

(1) A calibration curve is generated by adding ^{3}H-Flurazepam in 0.1 ml of buffer containing 0.03-0.2 ng range of flurazepam in buffer,

(2) Following preparation of the standards, duplicate portions of the reconstituted unknown flurazepam fractions are added to tubes containing ^{3}H-Flurazepam,

(3) Diluted antiserum is added to all tubes except the non-specific-binding control specimen to which buffer is added,

(4) The contents of each tube is mixed gently on a Vortex Mixer and allowed to stand at room temperature,

(5) Following incubation, the antibody-bound radio ligand is separated from the unbound fraction by precipitation with saturated ammonium sulphate,

(6) After the pellet is dissolved in water add 3 ml of scintillation fluid to produce a clear solution, and

(7) The radioactivity in each tube is quantified in a modified scintillation liquid counter.

RIA-Specificity* : The specificity of the antiserum initially is evaluated by cross-reactivity** studies involving all the **flurazepam metabolites** known to be present in plasma. The mono-as well as di-desethylmetabolites exhibited a cross-reactivity of 17 and 3.7% respectively, while other possible competitors cross-reacted less than 1% as shown in Table 32.1.

Table 32.1 : Flurazepam and Hapten 3-Hemisiccinyloxyl-flurazepam *vis-a-vis* the Cross-Reactivity of Metabolites Present in Plasma :

S.No.	Compound	Cross-Reactivity (%)
1.	Flurazepam	100
2.	3-Hemisuccinyloxyflurazepam	—
3.	3-Hydroxyflurazepam	—
4.	Monodesethylflurazepam	17.0
5.	Didesethylflurazepam	3.5
6.	N-1-Hydroxyethylflurazepam	1.0
7.	N-1-Desalkylflurazepam	1.0

Evidently, due to the cross-reactivity of both mono- and di-desethyl metabolites, a specific assay of flurazepam could not be developed successfully without first separating it from its metabolites effectively by the help of column chromatography.

32.5.5. RADIOIMMUNOASSAY OF CHLORDIAZEPOXIDE IN PLASMA

Chlordiazepoxide is the pioneer member of the **1, 4-benzodiazepines** to be employed clinically as an antianxiety agent in humans***. A number of methods based on extraction processes are available for the assay of this drug, namely : *spectrofluorometry*, *polarography* and *electron-capture GC-technique* ; but RIA measures it directly in the blood without involving extraction and possesses very low sensitivity.

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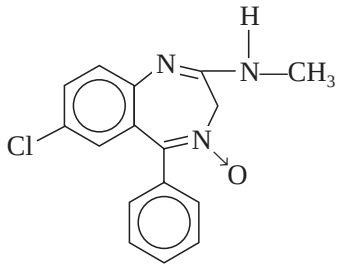
* **Specificity :** The degree of freedom from interference by substances other than the antigen.

** **Cross-reactivity :** The amount of a similar substance that will cause the same displacement of labelled antigen from the antibody as an arbitrary amount of the antigen. The usual definition is ID_{50}, which is the concentration of cross-reacting material required to displace 50% of labelled hapten from the antibody.

*** Harris, TH, **J. Amer. Med. Asso., 172,** 1162, 1960.



Chlordiazepoxide

Synthesis of Immunogen : Chlordiazepoxide as suspension in N-methylformamide is treated with HCl in dioxane to yield a pale-yellow solution. The resulting mixture is cooled to -30 °C and isoamyl nitrite in dioxane is added. The solution is stirred at – 30° to – 40 °C and aqueous ammonium sulfamate is added with continuous stirring.

The chilled azide solution is added slowly, dropwise with constant vigorous stirring into a solution of bovine-serum albumin. The pH is maintained at 8.0 to 8.7 by the careful addition of NaOH solution. The resulting pale-yellow solution is kept at 4°C for a duration of 36 hours and then dialysed against trimethamine buffer. After further dialysis for two days against distilled water, the immunogen is isolated by lyophilization.

Immunization and Antibody Production : The lypphilized immunogen obtained above is dissolved in normal saline and emulsified with equal volumes of complete Freund's adjuvant into a thick paste. Three New Zealand albino rabbits are immunized with the immunogen-paste through intradermal injections. The process is repeated twice at 2-weeks intervals followed by booster doses at monthly intervals. The antiserum is harvested when the plasma titer value is attained maximum.

RIA-Procedure : The various steps involved in the RIA procedure are enumerated below :

(1) A constant volume of control human plasma is added to a constant volume of each standard of chlordiazepoxide to produce a calibration curve of 2 to 100 ng per tube,

(2) The same volume of the unknown plasma samples is added to tubes containing constant volume of the solution of the labelled chlordiazepoxide and constant volume of the antiserum solution is now added to all the tubes,

(3) The volumes in all the tubes are made upto 1 ml with buffer solution, mixed thoroughly on a Vortex Mixer, and each tube is immersed in an ice-water bath,

(4) An equal volume of saturated ammonium sulphate solution is added to enable complete precipitation of globulin-bound chlordiazepoxide ^{14}C,

(5) After mixing the contents of the tubes thoroughly on a Vortex Mixer and allowing them to stand for a while at 4°C, the tubes are centrifuged at 3000 rpm,

(6) The supernate thus obtained containing unbound chlordiazepoxide-^{14}C is decanted into a counting vial and toluene is added, and

(7) The radioactivity in the supernate and that in the precipitate are separately counted in a scintillation counter.

Specificity of Antibody binding of Chlordiazepoxide : A good number of benzodiazepines are tested for their ability to complete with labelled chlordiazepoxide for the respective antibody binding site. The various competitors are adequately tested at a concentration of 200 ng *i.e..*,10-times the concentration of chlordiazepoxide required to produce a 50% inhibition of binding as shown in Table 32.2.

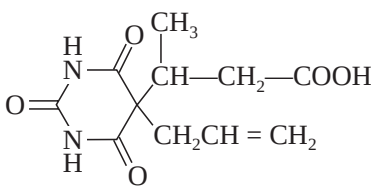
Table 32.2 : Specificity of Antiserum for Chlordiazepoxide*

S.No.	Compound	Cross-Reactivity (%)
1.	Chlordiazepoxide	100
2.	N-Desmethylchloriazepoxide	5
3.	Demoxepam	<1
4.	N-Desmethyldiazepam	<1
5.	Diazepam	<1
6.	Clonazepam	<1

From table 32.2 it is evident that the highest cross-reaction is 5% with N-desmethylchlordiazepoxide while demoxepam, N-desmethyldiazepam, diazepam and clonazepam displayed less than 1% inhibition. However, the RIA method appears to be reliable over a range of 2-100 ng per tube of chlordiazepoxide and, therefore, the sensitivity limit stands at 20 ng ml^{-1} using a 1.0 ml sample of plasma.

32.5.6. RADIOIMMUNOASSAY OF BARBITURATES

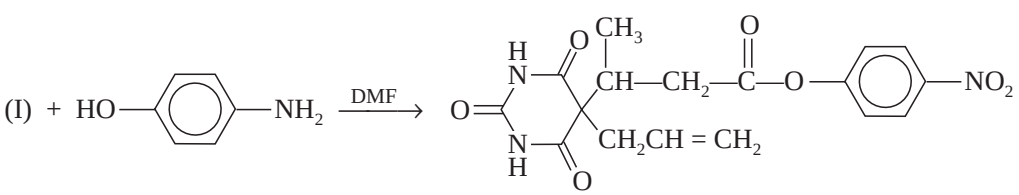
Barbiturates represent a class of **sedative and hypnotic drugs** employed extensively in medicine. RIA provides a rapid, sensitive specific and reliable means for their determination in plasma levels upto 5 ng without indulging in any type of extraction, filtration or evaporation as required for other conventional analytical methods**.



(I)

5-Allyl-5-(1-carboxyisopropyl) barbituric acid.

Synthesis of Immunogen (Hapten) : The barbiturate, 5-allyl-5-(1-carboxyisopropyl) barbituric acid (I) is first converted to 5-allyl-5-(1-*p*-nitrophenyloxycarbonylisopropyl) barbituric acid (II) by the interaction of the base with *p*-nitrophenol in N, N-dimethylformamide (DMF) as shown below :



p-Nitrophenol (II)

The resulting product (II) is subsequently coupled to bovine-serum-albumin in a glycerol-water mixture in the presence of dicyclohexylcarbodiimide. The mixture is incubated overnight at 4°C, and the protein-hapten complex is dialysed against distilled water thereby causing its purification. Conjugation of the respective barbiturate to the protein carrier, comparison of the barbiturate BGG-conjugate to control BGG-solution and preparation of ^{14}C-pentobarbital sodium are carried out respectively.

Preparation of Antiserum : The barbiturate-bovine-serum-albumin conjugate is duly emulsified with an equal volume of complete Freund's adjuvant and New Zealand albino rabbits are subsequently im-

* Dixon, WR, J. Earley and E. Postma, **J. Pharm. Sci., 64,** 937, 1975.

** Utiger, RD, **J. Clin. Invest., 44,** 1277, 1965.

munized with this particular emulsion. Six weeks after the initial does, booster doses are administered to the animals in each of their foot pads. Blood samples are collected 5-7 days after the booster injections and the serum is examined for antibodies to barbiturates. The antiserum is harvested when the serum antibody titer has attained its maximum level.

It has been observed that while normal, rabbit serum failed to bind labelled phenobarbital, the serum from immunized rabbits bound 75 to 80% of the added pentobarbital and there exists a linear relationship between ^{14}C-phenobarbital and the concentration of added antibody. Besides, when variable quantities of ^{14}C-pentobarbital are added to a constant quantity of antibody, there exists a linear relationship between added and bound ^{14}C-phenobarbital as depicted in Figure 32.4.

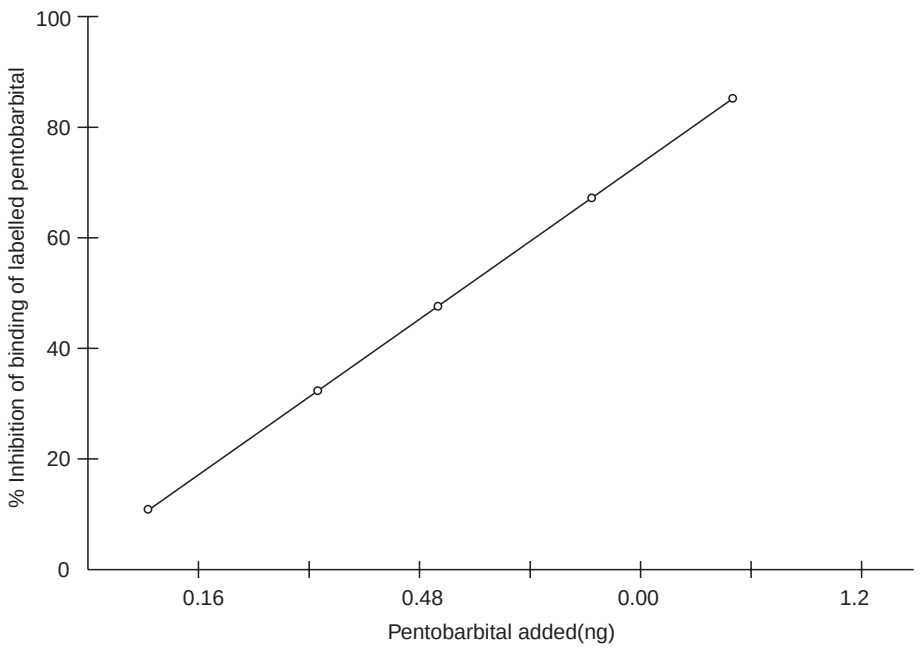
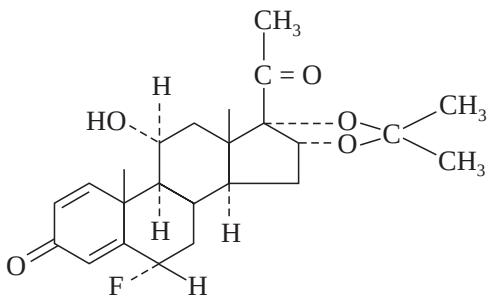


Figure 32.4 : Percentage Inhibition of Labelled Phenobarbital Binding by Added Unlabelled phenobarbital.

32.5.7. RADIOIMMUNOASSAY OF FLUNISOLIDE IN HUMAN PLASMA

Flunisolide is a fast-acting corticoid designed for the treatment of allergic rhinitis, asthma, and other allied respiratory disorders in humans*. As the quantum of drug delivered by inhalation (*i.e.*, the usual route of administration of the drug), is invariably small, the plasma-levels attained can also be fairly small. Hence, there is a dire need for a sensitive method of plasma concentration evaluation which is satisfied by radioimmunoassay.



Flunisolide

* Turkeltaub, PC, PS Norman, and S. Crepea, **J. Allergy Clin. Immunol., 55,** 120, 1975
Lowel, FC, JL Ohman. and M. Williams, **J. Allergy Clin. Immunol., 57,** 257, 1876.

Synthesis of Hapten Immunogen and Antiserum Production : The hapten, flunisolide-bovine-serum-albumin conjugate is prepared by coupling the 21-hemisuccinate of flunisolide to bovine-serum-albumin with a water-soluble carbodiimide coupling reagent*. The reaction mixture is dialysed exhaustively against normal saline to cause purification and the extent of conjugation is estimated by measuring the protein concentration**. However, the flunisolide residues are determined by UV-absorption method.

An emulsion of the hapten (*i.e.*, conjugate) in normal saline is prepared by mixing with an equal volume of Freund's complete adjuvant. The prepared emulsion is injected subcutaneously into four different sites in New Zealand albino rabbits. Six weeks after the initial injection, all the animals are placed on a regimen of weekly booster shots. After a period of six months, antiserum from these animals are harvested and dilutions of 1 : 10,000 to 1 : 30,000 produced 50% binding or more and is employed in the RIA.

RIA-Procedure : The following steps are to be adopted in a sequential manner, namely :

(1) Flunisolide standards required for the preparation of the standard curve are obtained by dilution of a stock solution of 10 mg of it in 10 ml of ethanol,

(2) A series of standard solution *viz*., 20, 50, 100, 200, 300, 500 and 600 pg per 0.1 ml in tris-(hydroxymethyl)-aminomethane/hydrochloric acid buffer and stored duly at 0 °C temperature,

(3) An ethanolic solution of ^{3}H-Flunisolide is diluted with tris-(hydroxymethyl)-aminomethane/hydrochloric acid buffer and 0.1% gelatin such that 0.1 ml portion contains 8,000 to 10,000 cmp activity,

(4) The antiserums are diluted in the said buffer with 0.1% gelatin to give rise to a total binding of between 35-50%,

(5) The charcoal stock solution is diluted as and when required with the aforementioned buffer immediately before, use,

(6) RIA is conducted by mixing together various dilutions of antiserum, buffer solution, ^{3}H-Flunisolide and various dilutions of flunisolide standard solutions in a set of test tubes,

(7) A second set of test tubes containing various dilutions of antiserum, buffer solution, ^{3}H-Flunisolide and various dilutions of the plasma being analysed of flunisolide content are prepared separately,

(8) The two sets of test tubes are incubated at temperature of 0 °C after adding constant volume of charcoal suspension to each of the tubes and mixing them thoroughly on a Vortex Mixer,

(9) The incubation is done overnight,

(10) The tubes are then centrifuged at 2500 rpm for 4 minutes and immediately 0.5 ml of the supernate is transferred into scintillation vials, and

(11) The scintillation fluid is added and the solutions are counted for 10 minutes in Scintillation Counter***.

The percentage inhibition is calculated and the values obtained from the first set of tubes is used to plot a standard curve. The concentrations of flunisolide from the standard curve values from their calulated percentage inhibition value as depicted in figure 32.5 below :

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* Sheenhan, JC and JJ Hlarka, **J. Org. Chem., 21,** 439, 1956.

** Lowry, OH, JJ Rosenbrough, AL Parr, and R. Randall., **J. Biol. Chem., 193,** 265, 1951.

*** Nerenburg, C., and SB Matin., **J. Pharm. Sci., 70,** 900, 1981.

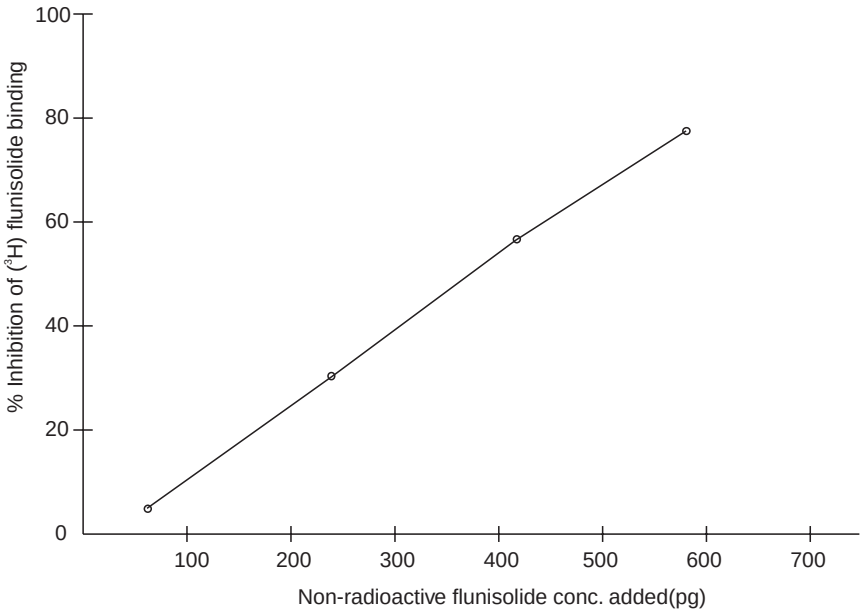


Figure 32.5 : Standard Curve (Displacement Curve) of Tritiated Flunisolide.

32.6. NOVEL APPLICATIONS OF RIA-TECHNIQUES

The radioimmunoassay technique has been gainfully exploited in a variety of novel applications of which only the *two* important aspects stated below will be discussed briefly, namely :

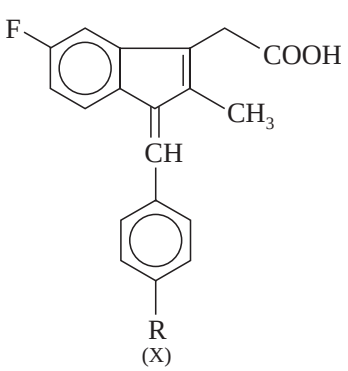
(*i*) Combined RIA Technique-Isotope Dilution, and

(*ii*) Stereospecificity.

32.6.1. COMBINED RIA-TECHNIQUES ISOTOPE DILUTION

In normal RIA-procedures the labelled drug or metabolite not only serves as the tracer for recovery but also for RIA quantification. However, the isotope dilution method categorically makes a clear separation of the drug and its metabolites. Consequently, a non-specific antiserum is employed to actually quantify the total amount of both unlabelled and labelled substance present.

The combined RIA-technique and isotope dilution has been successfully developed to estimate **SULINDAC*** along with its two prominent metabolites, namely : its sulphone and its sulphide present in the plasma-level as shown in the following chemical structures X and Y.

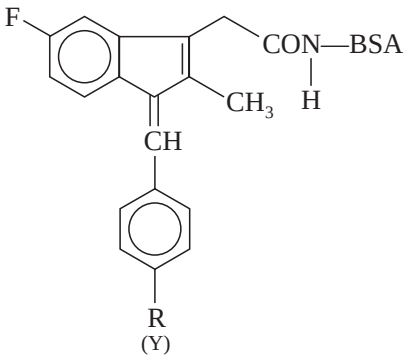


(X)

R	COMPOUND
$SOCH_3$	Sulindac
SCH_3	Sulindac Sulphide
SO_2CH_3	Sulindac Sulphone

[Sulindac and its metabolites]

* Hare, LE, CA Ditzler, M. Hichens, A. Rosegay and DE Duggan., **J. Pharma. Sci., 66,** 414, 1977.



R	COMPOUND
$SOCH_3$	Sulindac
SCH_3	Sulindac Sulphide
SO_2CH_3	Sulindac Sulphone

[Sulindac-Bovine-Serum-Albumin (BSA)]

CONJUGATE STRUCTURES

After due corrections have been incorporated with regard to recovery, it is possible to quantify the amount of standard sulindac or unknown ; besides standard metabolite or unknown.

Advantages : The various advantages are as follows :

(*i*) The methodology is not only very specific but also fairly sensitive, and

(*ii*) It serves as a substitute to simple RIA-procedure when specific antisera are not readily accessible.

Disadvantages : It has *two* main disadvantages :

(*i*) The method is time-consuming, and

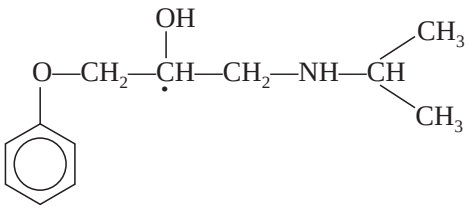
(*ii*) It involves tiresome and meticulous process of isotope dilution.

32.6.2. STEREOSPECIFICITY

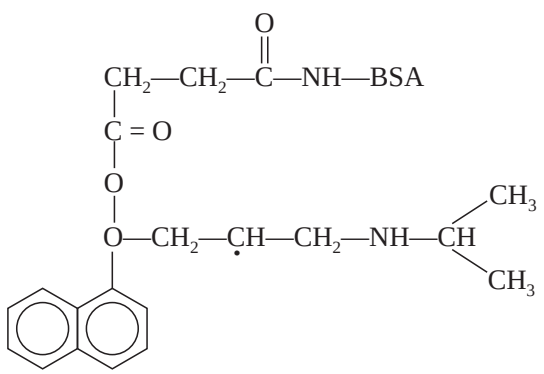
The **stereospecificity** of antigen-antibody reactions has gained its due recognition more than half-a-century ago*. However, an intensive and extensive stereospecificity radioimmunoassay procedures have been adequately applied to a number of pharmaceutical substances since mid-seventies, for instance : *atropine, propranolol, methadone*-to name a few.

Propranolol** which represents a comparatively better conceived example shall be discussed briefly as under with regard to its stereospecificity :

Propranolol is a recemic mixture *i.e.*, it contains an equimolecular portion of *d*- and *l*- isomers as given below :



Propranolol



Propranolol-Hemisuccinate-Bovine-Serum-Albumin (BSA) Conjugate

[• It shows the assymmetric carbon atom.]

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* Landsteiner, K., **'The Specificity of Serological Reactions',** Combridge, Harvard University Press, p. 168, 174, 1945.

** Kawashima, K., A. Levy and S. Spector., **J. Pharmaeol. Exp. Ther., 196,** 517, 1976.

Interestingly, only the *l*-propranolol exhibit β-blocking activity. In actual practice, two antisersa have been developed experimentally, namely :

(*a*) Antisera against the *dl*-racemic mixture, and

(*b*) Antisera against the *l*-isomer (active form only).

The *dl*-propranolol antiserum exhibits an almost equal affinity for both *d*- and *l*-isomers ; whilst the *l*-propranolol shows exclusively a marked and pronounced affinity for the *l*-isomer, By the application of these two RIA-techniques, it was practically feasible to quantify the plasma and heart concentrations of *dl*- and *l*-propranolol individually. Thus, the concentrations of *d*-propranolol could be arrived at by subtracting the concentration of *l*-isomer from the *dl*-mixture. It has been clearly demonstrated by Kawashima and coworkwers* that the *d*-propranolol undergoes distribution *in vivo* very sluggishly besides being metabolized more rapidly whereas the *l*-isomer gets distributed rather quickly to various tissues including the heart.

The RIA-procedure for propranolol is solely based on antisera derived from conjugates through the asymmetric carbon (*i.e.*, the optical carbon) as shown in the above chemical structures. Perhaps it could be possible that the stereospecificity of propranolol is caused due to the conformation of the drug-hapten in relation to the carrier protein to a great extent, through this hypothesis remains to be ascertained scientifically.

Consequently, the stabilization of the optical carbon by virtue of the conjugation to respective protein might improve upon the status of the specificity to a considerable extent. In order to prove the validity of this phenomenon one may carry out a definitive methodology whereby a closely monitored and controlled study of the antisera obtained by conjugates specifically prepared at the 'asymmetric-carbon' and at 'another-site' are both compared simultaneously under identical experimental parameters.

THEORETICAL AND PRACTICAL EXERCISES

1. What is Radioimmunoassay (RIA) ? Discuss its '*merits*' and '*demerits*' articulately.

2. Elaborate the theoretical aspects of RIA with suitable examples/explanations.

3. Discuss the various steps that are sequentially adopted in the **'Methodology of RIA'.**

4. How would you perform the assay of **'drugs'** by RIA as listed below ? Give structures, equations wherever applicable to strengthen your answer :

(*a*) Morphine,

(*b*) Hydromorphons and hydrocordone in Human Plasma,

(*c*) Clonazepam,

(*d*) Flurazepam in Human Plasma,

(*e*) Chlordiazepoxide in Plasma,

(*f*) Barbiturates, and

(*g*) Flunisolide in Human Plasma.

5. Give a brief account on the **'Novel Applications of RIA-Techniques'.** Support your answer with appropriate examples.

RECOMMENDED READINGS

1. Hayes, R., F. Goswitz and B. Murphy, Eds., '**Radioisotopes in Medicine : *In Vitro* Studies',** 1968.

2. Odell, WD and WH Daughaday, Eds., '**Principle of Competitive Proteinbinding Assays',** Philadelphia, Lippincott, 1971.

* Kawashima, K., A. Levy and S. Spector., **J. Pharmaeol. Exp. Ther., 196,** 517, 1976.

3. Davies, DS and BNC Prichard, Eds., '**Biological Effects of Drugs in Relation to their Plasma Concentration',** New York, McMillan, 1973.

4. '**Radioimmunoassay and Related Procedures in Medicine'** Vols 1 and 2., Vienna, International Atomic Energy Agency, 1974.

5. Croll, MN, LW Brady, T. Honda and RJ, Waliner., Eds., '**New Techniques in Tumour Localization and Radioimmunoassay',** New York, Wiley 1974.

6. Mule, SI, I. Sunshine, M. Braude and RE Willeta, Eds' '**Immunoassays for Drugs Subject to Abuse',** Cleveland Inc., Ohio CRC-Press, 1974.

7. Gennaro AR (ed.) : **Remington : The Science and Practice of Pharmacy**, 20th edn. Vol. I & II, Lippincott Williams & Wilkins, New York, 2004.