Thermoanalytical methods essentially encompass such techniques that are based entirely on the concept of heating a sample followed by well-defined modified procedures, such as: gravimetric analysis, differential analysis and titrimetric analysis. In usual practice, data are generated as a result of continuously recorded curves that may be considered as ‘thermal spectra’. These thermal spectra also termed as ‘thermograms, often characterize a single or multicomponent system in terms of:

(a) temperature dependencies of its thermodynamic properties, and

(b) physicochemical reaction kinetics.

Broadly speaking the thermoanalytical methods are normally classified into the following three categories, namely:

(i) Thermogravimetric Analysis (TGA),

(ii) Differential Thermal Analysis (DTA), and

(iii) Thermometric Titrations.

All the above mentioned techniques shall be discussed briefly with specific reference to their theory, instrumentation, methodology and applications wherever necessary.

Differential Thermal Analysis (DTA)
The difference of temperature between the sample under estimation and a thermally-inert reference material is continuously recorded as a function of furnace temperature in differential thermal analysis (DTA).

**DIFFERENTIAL THERMAL ANALYSIS (DTA)**

1. **THEORY**

The difference of temperature between the sample under estimation and a thermally-inert reference material is continuously recorded as a function of furnace temperature in differential thermal analysis (DTA). In actual practice both TGA and DTA are regarded as complementary techniques whereby information gathered by the usage of one approach is invariably supplemented and enhanced by the application of the other method. The range of phenomena measurable during a DTA-run is found to be much larger than in a TGA-run.

It is pertinent to mention here that in the course of TGA many vital processes, for instance: crystallization, crystalline transitions, pure fusion reactions, glass transitions, and solid-state reactions devoid of volatile components might not be indicated as they happen to cause little change in weight of the sample. TGA invariably describes with ample precision the stoichiometry related to chemical changes that are indicated during DTA by an endothermal or exothermal duration from the base-line.

2. **INSTRUMENTATION**

A differential thermal analyzer is composed of five basic components, namely:

(i) Sample holder with built-in thermocouple assembly,

(ii) Flow-control system,
(iii) Furnace assembly,
(iv) Preamplifier and Recorder, and
(v) Furnace Power Programmer and Controller.

A typical commercial differential thermal analyzer is schematically illustrated in Figure 11.3.

(a) Thermocouples employed are normally unsheathed Platinum Vs Platinum and Sodium Vs 10% Rhodium. The said two thermocouples help in measuring the difference in temperature between a sample S and an absolutely inert reference substance R, as both are subjected to heating in a ceramic or metal block inside a furnace being operated by a temperature programmer and controller.

(b) The output of the differential thermocouple is amplified adequately through a high gain, low-noise preamplifier and subsequently hooked to the recorder, one axis of which is driven by the block temperature signal and is measured by a third thermocouple.

(c) Heating/Cooling Device : A sufficient versatility is achieved by the aid of a pressure-vacuum, high-temperature electric furnace. An almost constant heating rate is usually achieved by using a motor-driven variable auto transformer.

Both heating rates and cooling rates may be conveniently adjusted continuously :

(i) From 0°-30°C/minute by some instruments, and
(ii) From a choice of several commonly employed heating rates viz., 2°, 4°, 8° and 16°C/minute.

Usual workable sample temperatures are upto : 500°C. Exceptional maximum temperatures are upto : 1000°C.

(d) Relatively small sample volumes help in two ways : first, they make evacuation easy ; and secondly, they minimize thermal gradients.

3. METHODOLOGY

(i) Insert a very thin thermocouple into a disposable sample tube 2 mm in diameter and containing 0.1-10 mg of sample,

(ii) Another identical tube is either kept empty or filled with a reference substance, such as quartz, sand, alumina or alundum powder,

(iii) The two tubes are simultaneously inserted into the sample block and subsequently heated (or cooled) at a uniform predetermined programmed rate, and

(iv) DTA—being a dynamic process, it is extremely important that all aspects of the technique must be thoroughly standardised so as to obtain reproducible results. A few of these aspects vital aspects are :

. Pretreatment of the specimen,
· Particle size and packing of the specimen,
· Dilution of the specimen,
· Nature of the inert diluent,
· Crystalline substances must be powdered, and sieved through 100-mesh sieve,
· For colloidal particles (e.g., clays), micelle-size is very critical, and
· Either to suppress an unwanted reaction (e.g., oxidation), or to explore the study of a reaction (e.g., gaseous reaction product)—the atmosphere should be controlled adequately.

Figure 11.4, depicts the differential thermal analysis investigation of calcium acetate monohydrate at a uniform programmed heating rate of 12°C/minute..

![Diagram](image)

The chemical reactions involved in the differentiated thermal analysis of calcium acetate monohydrate may be expressed as follows:

\[
\begin{align*}
\text{Ca(CH}_2\text{COO)}_2\text{H}_2\text{O} & \quad \text{200-250°C} \quad \text{Ca(CH}_2\text{COO)}_2 \quad \text{350-400°C} \quad \text{CaCO}_3 \quad \text{800-950°C} \\
(\text{Stage-I}) & \quad \text{Ca(OH)}_2 \quad \text{(Stage-II)} & \quad \text{CaO} \quad \text{(Stage-III)}
\end{align*}
\]

Stage I : The endothermal dehydration of calcium acetate monohydrate occurs giving rise to the anhydrous salt. It is easily noticed by an endothermal band on DTA curve between 200°C and 250°C.

Stage II : The anhydrous salt undergoes endothermal decomposition reaction at 350-400°C resulting into the formation of CaCO3. It has been observed that this decomposition reaction seems to be almost alike in the presence of either CO2 or Ar.

Stage III : The decomposition of calcium carbonate to calcium oxide, which is a function of the partial pressure of the CO2 in contact with the sample. The endothermal band for the carbonate decomposition is sharply peaked spread over a relatively narrower temperature range in an atmosphere of CO2.

4. APPLICATIONS
The various important applications of DTA are:

(i) Rapid identification of the compositions of mixed clays,

(ii) Studying the thermal stabilities of inorganic compounds,

(iii) Critically examining in a specific reaction whether a new compound is actually formed or the product is nothing but an unreacted original substance, and

(iv) DTA offers a wide spectrum of useful investigations related to reaction kinetics, polymerization, solvent retention, phase-transformations, solid-phase reactions and curing or drying properties of a product.

Thermogravimetric Analysis (TGA): A large number of chemical substances invariably decompose upon heating, and this idea of heating a sample to observe weight changes is the underlying principle of thermogravimetric analysis (TGA).
HERMOGRAVIMETRIC ANALYSIS (TGA)

1. THEORY

A large number of chemical substances invariably decompose upon heating, and this idea of heating a sample to observe weight changes is the underlying principle of thermogravimetric analysis (TGA). However, TGA may be sub-divided into two heads, namely:

(a) Static (or Isothermal) Thermogravimetric Analysis, and

(b) Dynamic Thermogravimetric Analysis.

1.1. Static Thermogravimetric Analysis

In this particular instance the sample under analysis is maintained at a constant temperature for a period of time during which any changes in weight are observed carefully.

1.2. Dynamic Thermogravimetric Analysis

In dynamic thermogravimetric analysis a sample is subjected to conditions of predetermined, carefully controlled continuous increase in temperature that is invariably found to be linear with time.

2. INSTRUMENTATION

The essential requirements for an instrument (Figure 11.1) meant for thermogravimetric analysis are, namely:

(a) A high-precision balance,

(b) A furnace adequately programmed for a linear rise of temperature with time, and
2.1. Balances

They are usually of two types:

(a) Null-point Type: It makes use of an appropriate sensing-element which aptly detects any slightest deviation of the balance beam and provides the application of a restoring force, directly proportional to the change in weight, thereby returning the beam to its original null-point. The restoring-force is subsequently recorded either directly or with the aid of a transducer.

(b) Deflection Type: It is essentially based on either a conventional analytical balance consisting of helical spring, cantilever beam and strain gauze or a torsion analytical balance involving the conversion of deviations directly into a record of the weight change.

2.2. Furnace

The furnace must be designed in such a fashion so as to incorporate an appropriate smooth input thereby maintaining either a fixed temperature or a predetermined linear-heating programme (e.g., 10°C-600°C per hour).

The temperature control of the furnace is satisfactorily achieved via a thermocouple mounted very close to the furnace-winding. The maximum operational temperature may be obtained by using different thermocouples as indicated below:
2.3. Recorder

The recording device must be such so as to:

(i) record both temperature and weight continuously, and

(ii) make a definite periodic record of the time.

3. METHODOLOGY

The ‘thermogram’ for calcium oxalate monohydrate (CaC₂O₄·H₂O) is presented in Figure 11.2. The successive plateaus correspond to the anhydrous oxalate (100-250°C), calcium carbonate (400-500°C), and finally calcium oxide (700-850°C). In other words, these plateaus on the decomposition curve designate two vital aspects, namely:

(a) clear indication of constant weight, and

(b) stable phases within a specified temperature interval. The chemical reactions involved may be summarized as follows:

3.1. Interpretation of Thermogram

In the thermogram (Figure 11.2), which vividly illustrates the thermogravimetric evaluation of CaC₂O₄·H₂O, it is ensured that the weight of this product decreases in several stages, namely:

Stage 1: The water of hydration (or crystallization) from calcium oxalate monohydrate is lost which corresponds to 2.46 mg (12.3%) equivalent to 1 mole of H₂O in the temperature range 100-250°C.
Actually, the 12.3% weight loss that took place between 100-250°C should correspond to 12.3% of the original formula weight for CaCO$_3$H$_2$O (FW = 146). Hence, the product being lost has a formula weight of $0.123 \times 146 = 17.958$ (~ 18.0), and it corresponds to H$_2$O.

Stage 2: One mole of carbon monoxide is evolved subsequently from calcium oxalate, corresponding to 3.84 mg (19.2%) in the temperature range 400-500°C.

The 19.2% weight loss that occurred between 400-500°C should correspond to 19.2% of the original formula weight of 146. Therefore, the product being given out has a formula weight of $1.92 \times 146 = 28.0$, that corresponds to CO.

Stage 3: Finally, a mole of CO$_2$ is evolved from calcium carbonate that corresponds to 6.02 mg (3.01%) in the temperature range 700-850°C.

The weight loss amounting to 3.01% which took place in the range 700-850°C must, in fact, corresponds to 3.01% of the original formula weight of 146. Therefore, the product being evolved has a formula weight of $0.301 \times 146 = 43.946$ (~ 44), and it corresponds to CO$_2$.

It is quite evident that in a multicomponent system wherein more than one component exhibits weight variations and that too at different temperature regions, the composition of the original compound may be estimated as depicted in Figure 11.2.

In a situation whereby an inert material is present along with a pure substance, from the generated thermogram the composition of the mixture may be derived from the percentage weight variation which takes place relative to the percentage weight variation observed with the pure compound (A), by employing the following expression:

$$\text{Component A (wt %)} = \frac{\% \text{ wt. change for mixture}}{\% \text{ wt. change for pure compound A}} \times 100 \%$$

4. APPLICATIONS

The most broadly based application of the thermogravimetric analysis (TGA) has been visualized and exploited in the investigation of analytical methods, such as:

(i) Determining appropriate forms for many elements,

(ii) Screening and testing of substances which may be used as potential analytical standards (primary standard), and

(iii) Direct application of the technique in analytical assays.

A few typical applications of TGA are, namely:

(a) Plateaus for hydrates are sometimes based on the initial water content (i.e., water of crystallization). It has been observed that in humidified air at low heating rates, hydrates usually give rise to good plateaus.

Example: Dehydration of sodium tungstate 28-hydrate [Na$_2$WO$_4$.28 H$_2$O (5 : 12)]
Experimental parameters*:
A. Humidified air, 300°C/hour,
B. Humidified air, 150°C/hour,
C. Humidified air 10°C/hour,
D. Room air, 10°C/hour,

Sample weight: 0.5000 g;

\[ n = \text{Moles water per } 5\text{Na}_2\text{O}, 12\text{WO}_3 \]

(b) Analysis of flue-gas scrubber system in environmental analysis.

The flue-gas emitted from a coal-fired-power-plant is subjected to scrubbing by the aid of wet limestone to get rid of sulphur dioxide (SO\(_2\)) as completely as possible. TGA helps in monitoring the system by carrying out the analysis of the products resulting from the scrubbing process, that mainly consist of (i) CaCO\(_3\); (ii) CaSO\(_3\) . CaSO\(_3\) . 1/4 H\(_2\)O, and (iii) CaSO\(_4\) . 2H\(_2\)O.

The thermogram obtained from TGA provides the following valuable informations which suggests the decomposition occurring at three distinct stages thereby causing the loss due to two moles of water, half-a-mole of water and one mole of CO\(_2\).

(c) The stepwise degradation of organic polymers has received adequate attention which has broadened the in-depth knowledge of polymer chemistry. In this specific instance the sample is either heated under vacuum or in an inert atmosphere (of N\(_2\)).

(d) The thermogravimetric data may be employed to evaluate the kinetic parameters of weight varia-tions in reactions.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Conversion From</th>
<th>Conversion To</th>
<th>Wt. Loss Region (°C)</th>
<th>Wt. Loss (%)</th>
<th>Due To</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>CaSO(_4) . 2H(_2)O</td>
<td>CaSO(_4)</td>
<td>100-200</td>
<td>66</td>
<td>2H(_2)O</td>
</tr>
<tr>
<td>2.</td>
<td>CaSO(_3) . CaSO(_3) . 1/4 H(_2)O</td>
<td>CaSO(_3) . CaSO(_3)</td>
<td>420</td>
<td>31</td>
<td>1/2H(_2)O</td>
</tr>
<tr>
<td>3.</td>
<td>CaCO(_3)</td>
<td>CaO</td>
<td>630-800</td>
<td>03</td>
<td>CO(_2)</td>
</tr>
</tbody>
</table>

LIQUID-LIQUID EXTRACTION

INTRODUCTION

Liquid-Liquid extraction is a versatile and dependable separation technique wherein an aqueous solution is usually brought into contact with another organic solvent, exclusively immiscible with the former, so as to affect a legitimate and actual transfer of either one or more solutes into
the latter. The normal-feasible separations which can thus be achieved are found to be rather easy, fast, convenient and effective reasonably. Invariably such separations may be performed by shaking the two liquids in a separatory funnel for a few minutes; and may be extended either to large quantities of pharmaceutical substances or trace levels.

In the case of pharmaceutical chemicals that are mostly ‘organic solutes’, the liquid-liquid extraction system may very often make use of two immiscible organic solvents (e.g., alcohol and ether) instead of the aqueous-organic type of extraction. On the contrary, the ‘inorganic solutes’ normally encountered are in-variably in aqueous solutions; therefore, it has become absolutely necessary to produce such neutral sub-stances out of them, for instance ion-association complexes and metal-chelates (using organic-ligands) that may be extracted into an appropriate organic solvent.

In short, liquid-liquid extraction has been employed predominantly and effectively not only for the pre-concentration and isolation of a ‘single’ chemical entity just before its actual estimation, but also for the extraction of classes of organic compounds or groups of metals, just prior to their usual estimation either by chromatographic techniques or by atomic-absorption methods.

Liquid-Liquid Extraction: Theory

1. Error Due to the Volume Change
2. Effectiveness of an Extraction

THEORY

The behavioural pattern of two immiscible solvents, say ‘a’ and ‘b’, is essentially nonideal with respect to one another. Now, if a third substance is made to dissolve in a two-phase mixture of the solvents (i.e., ‘a’ and ‘b’), it may behave ideally in either phases provided its concentration in each individual phase is approximately small. Therefore, under these prevailing experimental parameters the ratio of the mole fractions of the solute in the two respective immiscible phases (‘a’ and ‘b’) is found to be a constant which is absolutely independent of the quantity of solute present. It is termed as the Nernst Distribution Law or the Partition Law and may be expressed as follows:

\[
K_p = \frac{[A]_a}{[A]_b} = \frac{\text{Concentration of solute in solvent } 'a'}{\text{Concentration of solute in solvent } 'b'}
\]

\[...(a)\]

where, \([A]_a\) = Mole fraction of solute A in Phase ‘a’

\([B]_b\) = Mole fraction of solute B in Phase ‘b’, and

\(K_p\) = A constant.

The constant (Kp) is also known as the distribution coefficient or the partition coefficient. Interest-ingly, this particular relation [Eq. (a)] was originally derived for ideal solutions only, but it caters for a fairly good description of the behavioural pattern of a number of real-extraction systems encountered in the analy-sis of pharmaceutical substances. However, the Partition Law offers the following two limitations, namely:
(a) It is not thermodynamically rigorous i.e., it takes no cognizance of the activities of the different species. In other words, it is solely applicable to very dilute solutions in which case the ratio of the activities almost approaches unity, and

(b) It does not hold good when the distributing substances encounters association or distribution in either phases (i.e., ‘a’ and ‘b’).

Consequently, a more rigorous treatment particularly specifies $K_p$ as the ratio of the activities of the substance (A) in the two solvents instead of their concentrations. Hence, for dilute solutions, at a specified constant pressure and temperature, the mole fraction of a solute is directly proportional to its concentration in molarity or mass per unit volume; which implies that these may be employed instead of mole-fraction in Eq. (a).

Thus, the Partition Coefficient $K_p$ is also given by the following expression:

$$K_p = \frac{S_1}{S_2}$$

..............................(b)

where, $S_1 = \text{Solubility of substance A in solvent ‘a’}$, and

$S_2 = \text{Solubility of substance A in solvent ‘b’}$.

Adequate precaution and care must be exercised in determining partition coefficients based on the solubility data as $S_1$ is not the solubility of substance ‘A’ in pure Solvent ‘a’, but rather the solubility in Solvent ‘a’ saturated with Solvent ‘b’.

Example: In order to determine the exact partition coefficient of substance ‘A’ between water and ethyl acetate, the appropriate solubilities would be those of the substance ‘A’ in 3.3% ethyl acetate in water (composition of the ‘aqueous’ layer) and 8.7% in water in ethyl acetate (composition of the ‘ester’ layer).

Likewise, the following Table 27.1, records the mutual solubilities of a few typical solvent pairs that are used frequently for liquid-liquid extraction procedures.
In liquid-liquid extractions the following two aspects are very crucial and important, namely:

(a) Error due to the Volume Change, and

(b) Effectiveness of an Extraction.

These two aspects shall be discussed briefly at this juncture.

1. ERROR DUE TO THE VOLUME CHANGE

In a situation wherein two immiscible solvents are employed in an extraction, the volumes of the two individual phases after attainment of equilibrium may be appreciably different in comparison to the initial volumes of the solvents used. Therefore, a number of procedures have been adopted to avoid ‘error due to the volume change’ incurred thereby, namely:

(i) Measure the volume of the phase employed for the analysis and incorporate this volume in the calculations,

(ii) Separate the phase quantitatively and subsequently dilute to a known volume,

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Solvent Pair</th>
<th>Percentage Composition Phases</th>
<th>Composition of Azeotrope (%)</th>
<th>Boiling Pt. of Azeotrope (°C)</th>
<th>Boiling Pt. of Solvents (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Upper</td>
<td>Lower</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>1-Butanol-</td>
<td>79.9</td>
<td>7.7</td>
<td>55.5</td>
<td>93.0</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>20.1</td>
<td>92.3</td>
<td>44.5</td>
<td>100.0</td>
</tr>
<tr>
<td>2.</td>
<td>n-Butyl ether-</td>
<td>99.97</td>
<td>0.19</td>
<td>66.6</td>
<td>94.1</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>0.03</td>
<td>99.81</td>
<td>33.4</td>
<td>100.0</td>
</tr>
<tr>
<td>3.</td>
<td>Carbon tetrachloride-</td>
<td>0.03</td>
<td>99.97</td>
<td>95.9</td>
<td>66.8</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>99.97</td>
<td>0.03</td>
<td>4.1</td>
<td>100.0</td>
</tr>
<tr>
<td>4.</td>
<td>Chloroform-</td>
<td>0.8</td>
<td>99.8</td>
<td>97.0</td>
<td>56.3</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>99.2</td>
<td>0.2</td>
<td>3.0</td>
<td>100.0</td>
</tr>
<tr>
<td>5.</td>
<td>Dichloromethane-</td>
<td>2.0</td>
<td>99.9</td>
<td>99.0</td>
<td>38.8</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>98.0</td>
<td>0.1</td>
<td>1.0</td>
<td>100.0</td>
</tr>
<tr>
<td>6.</td>
<td>Ethyl ether-</td>
<td>98.53</td>
<td>6.04</td>
<td>98.2</td>
<td>34.2</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>1.47</td>
<td>93.96</td>
<td>1.2</td>
<td>100.0</td>
</tr>
<tr>
<td>7.</td>
<td>Hexane-</td>
<td>85.0</td>
<td>42.0</td>
<td>73.1</td>
<td>69.0</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>15.0</td>
<td>58.0</td>
<td>26.9</td>
<td>64.7</td>
</tr>
<tr>
<td>8.</td>
<td>1-Octanol-</td>
<td>—</td>
<td>0.1</td>
<td>10.0</td>
<td>99.4</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>—</td>
<td>99.9</td>
<td>90.0</td>
<td>100.0</td>
</tr>
<tr>
<td>9.</td>
<td>Toluene-</td>
<td>99.95</td>
<td>0.06</td>
<td>79.8</td>
<td>85.0</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>0.05</td>
<td>99.94</td>
<td>20.2</td>
<td>100.0</td>
</tr>
<tr>
<td>10.</td>
<td>m-Xylene-</td>
<td>99.95</td>
<td>0.05</td>
<td>60.0</td>
<td>94.5</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>0.05</td>
<td>99.95</td>
<td>40.0</td>
<td>100.0</td>
</tr>
</tbody>
</table>
(iii) Separate the phase quantitatively and make use of the entire volume in the remaining steps of the ongoing analysis, and

(iv) Carry a marker substance through the extraction to automatically compensate for volume changes.

However, the latter procedure finds its abundant use in chromatographic methods of analysis.

2. EFFECTIVENESS OF AN EXTRACTION

Based on the appropriate partition coefficient of an immiscible solvent pair it is possible to calculate the ‘effectiveness of an extraction’.

Let us assume that ‘x’ moles of solute present initially in a volume $V_2$ of Solvent ‘b’. Now, this particular sample undergoes extraction with a volume $V_1$ of Solvent ‘a’ and subsequently ‘y’ moles of compound are left in $V_2$ at equilibrium.

Substituting these values in Eq. (a) and using molarity instead of mole fraction, we have:

$$K_p = \frac{M_1}{M_2} = \left(\frac{x - y}{V_1}\right) \left/ \left(\frac{y}{V_2}\right)\right.$$

...\,(c)

after simplifying and rearranging:

$$K_p = \left(\frac{x}{V_1} - \frac{y}{V_2}\right) \frac{V_2}{y}$$

or

$$= \frac{x}{y} \frac{V_2}{V_1} - \frac{V_2}{V_1}$$

or

$$= \frac{V_2}{V_1} \left(\frac{x}{y} - 1\right)$$

or

$$K_p \cdot \frac{V_1}{V_2} = \frac{x}{y} - 1$$

or

$$K_p \cdot \frac{V_1}{V_2} + 1 = \frac{x}{y}$$

or

$$\frac{y}{x} = \left(\frac{V_1}{V_2} K_p + 1\right)^{-1} = f$$
where, \( f \) = Fraction not extracted.

Figure 27.1, represents the nomogram from which the unextracted fraction for various values of \( V_2/V_1 \) and \( K \) may be obtained.

From Eqs. \((d)\) it is quite evident that the fraction extracted is absolutely independent of the initial solute concentration. Hence, the fraction left unextracted after \( \text{'}n\text{' extraction} \) may be given by the following expression:

\[
fn = \left( \frac{V_1}{V_2} K_p + 1 \right)^{-n}
\]

...........................................(e)

assuming that the same volumes of solvents have been used for each extraction.

Form Eq. \((e)\) it is distinctly obvious that a series of several extractions would definitely prove to be more efficacious than one single extraction using the same total volume of solvent.

From figure 27.1, the following steps may be adopted in order to determine the percentage of the analyte left in the sample after a single extraction:

\((i)\) Hold a straight edge in such a manner that it is made to pass though the point on the ‘left scale’ which exactly corresponds to that ratio of extracting solvent to sample solvent volume and through the point representing the partition coefficient \( (K_p) \) on the ‘middle scale’, and

\((ii)\) The percentage of the ‘analyte’, left behind in the sample solvent after a single extraction is given by the intersection of the straight-edge with the ‘right scale’. 
FACTORS INFLUENCING SOLVENT EXTRACTION

A number of cardinal factors exert a positive influence on the phenomenon of solvent extraction, namely:

(a) Effect of temperature and inert solutes,

(b) Effect of pH on extraction,

(c) Effect of ion-pair formation, and

(d) Effect of synergistic extraction.

These factors shall be discussed briefly below:

1. EFFECT OF TEMPERATURE AND INERT SOLUTES

The physical as well as chemical interactions of a solute is capable of changing its apparent partition coefficient between a pair of solvents. Therefore, it is absolutely necessary to take this into consideration while selecting an appropriate extraction-system. Craig and Craig* have advocated that the partition coefficients are normally not sensitive to temperature when the two
solvents in question are more or less immiscible and also the concentrations are fairly low in both the phases. Thus, the effect of temperature on the partition coefficient may be estimated conveniently from its effect on the solubilities of the substance in the two respective solvents. By substituting the solubilities (e.g., $S_1$ and $S_2$) in Eq. (b) it is possible to estimate $K$.

The effect of inert solutes, such as: calcium chloride, magnesium chloride and sucrose, can also be employed judiciously and efficaciously in the development of solutions to difficult extraction problems by allowing efficient extractions from the water into such solvents as acetone, ethanol and methanol that are found to be completely miscible with water in the absence of salt. Matkovitch and Cristian* found the above three inert solutes to be the best agents for salting acetone out of water. It has been observed that the acetone layer that separated from a saturated aqueous solution of CaCl$_2$ exclusively contained 0.32 ± 0.01% water (v/v) and 212 ppm salt (w/w) at equilibrium.

2. EFFECT OF pH ON EXTRACTION

Generally, it has been found that the organic acids and bases do exist in aqueous solution as equilibrium mixtures of their respective neutral as well as ionic forms. Thus, these neutral and ionic forms may not have the same identical partition coefficients in a second solvent; therefore, the quantity of a substance being extracted solely depends upon the position of the acid-base equilibrium and ultimately upon the pH of the resulting solution. Hence, extraction coefficient (E) may be defined as the ratio of the concentrations of the substance in all its forms in the two respective phases in the presence of equilibria; and it can be expressed as follows:

$$E = \text{Extraction Coefficient} = \frac{\Sigma[S_i]_2}{\Sigma[S_i]_1} \quad \text{..........................(i)}$$

where, $\Sigma[S_i]_2$ = The sum total of all forms of the compound in Phase-‘2’, and
$\Sigma[S_i]_1$ = The sum total of all forms of the compound in Phase ‘1’.

In fact, the actual effect of the equilibrium on the extraction may be shown by determining the extraction coefficient for the system:

$$A + H \rightleftharpoons AH \quad \text{or} \quad K = \frac{[AH]_1}{[A]_1[H]_1} \quad \text{..........................(ii)}$$

where, $A$ = Extract with partition coefficient $K_p$, $A$ and
$AH$ = Extract with partition coefficient $K_p$, AH
Hence, \[ K_p, A = \frac{[A]_2}{[A]_1} \text{ and } K_p, AH = \frac{[AH]_2}{[AH]_1} \] ..........................(iii)

Therefore, for this particular system the efficiency coefficient \( E \) may be expressed as follows:

\[ E = \frac{[A]_2 + [AH]_2}{[A]_1 + [AH]_1} \] ..........................(iv)

Now, substituting Eq. (ii) and Eq. (iii) into Eq. (iv) and subsequently simplifying, we shall get:

\[
E = \frac{K_p, A[A]_1 K_p, AH[AH]_1}{[A]_1 + [AH]_1} \quad \text{From Eq. (iii)}
\]

or

\[
E = \frac{[A]_1 (K_p, A + K_p, AH[AH]_1) / [A]_1)}{[A]_1 ([A]_1 + [AH]_1) / [A]_1)}
\]

or

\[
E = \frac{K_p, A + K_p, AH K[H]_1}{1 + K[H]_1} \quad \text{From Eq. (ii)} \quad ...(v)
\]

From Eq. (v) it is quite evident that \( E \) approaches \( K_p, A \) as \( K[H]_1 \) becomes small and \( K_p, AH \) as \( K[H]_1 \) becomes large.

Now, assuming that only \( A \) extracts (i.e., \( A \) being a neutral organic base and \( AH \) the conjugate acid),

Eq. (v) may be expressed as:

\[
E = K_p, A \frac{1}{1 + K[H]_1} = K_p, A \frac{[A]}{[A] + [AH]} \]

...(vi)

The following inferences may be arrived at on the basis of Eq. (vi), namely:

(a) Extraction coefficient (\( E \)) is just the partition coefficient times the fraction of the analyte which is present in the extractable form,

(b) Under a given set of experimental parameters the ultimate effect of the ‘equilibrium’ shall be to reduce the amount extracted, and

(c) Forcibly shifting the ‘equilibrium’ toward the extractable species by adjusting the pH helps to minimise the effect of the equilibrium thereby rendering \( E \) almost equal to \( K_p, A \).

In conclusion, it may be observed that the pH for an ‘extraction system’ must be selected in such a fashion so that the maximum quantum of the analyte is present in the extractable form, that
obviously suggests that the analyte should always be in the form of either a free base or a free acid. From the actual practical experience it has been noticed that a good-working range lies between 95 to 97% present in the extractable form.

3. EFFECT OF ION-PAIR FORMATION

Ion-pair formation needs its due recognition because it very often gives rise to unexpected extractions. In true sense, ion-pair may be regarded as a close association of an anion and cation, and therefore, it usually takes place either in a polar or a non-polar solvent. In reality, the ion-pairs are invariably formed by virtue of the union between comparatively large organic anions and (much smaller) cations. Interestingly, the resulting ion-pairs have been found to show their appreciable solubility in polar solvents; and hence, these species may be extracted conveniently under such experimental parameters where neither individual component ion could.

A few vital criteria towards the formation of an improved aqueous extractable ionic species are, namely:

· Formation of a neutral metal-chelate complex or by ion association, and
· Creation of larger and more hydrophobic molecular species.

A few typical examples shall be discussed here to explain the chelate-formation:

Example 1: $\text{Cu}^{2+}$ with ‘acetylacetonate’ forms a fairly stable ring compound:

\[
\begin{align*}
\text{H}_3\text{C}-\text{C} &= \text{O} \quad &\text{H}_3\text{C}-\text{C} &= \text{O} \\
\text{H}_3\text{C}-\text{C} &= \text{OH} &\rightarrow &\text{H}_3\text{C}-\text{C} &= \text{O} \\
\text{H}_3\text{C}-\text{C} &= \text{O} &\rightarrow &\text{H}_3\text{C}-\text{C} &= \text{O} \\
\text{Cu}^{2+} &\rightarrow &\text{Cu}^{2+} &\rightarrow &\text{Cu}^{2+} \\
\text{Acetylacetonate} &\rightarrow &\text{Acetylacetonate} &\rightarrow &\text{Copper(II)-acetylacetone complex} \\
\text{(keto-form)} &\rightarrow &\text{(enol-form)} &\rightarrow &\text{(Resonating ring compound)}
\end{align*}
\]

Example 2: Iron (III) ‘cupferrate’ gives rise to a stable ring compounds as shown below:

\[
\begin{align*}
\text{NH}_4^+ &\rightarrow \text{NH}_4^+ \\
\text{N} &= \text{O} &\rightarrow &\text{N} &= \text{O} \\
\text{Fe}^{3+} &\rightarrow &\text{Fe}^{3+} \\
\text{Example 3: Sulphonic acids rapidly pair with a plethora of ‘protonated amines’ to form an easily extractable complex}
\end{align*}
\]
Example 4: Cl⁻ ion serves as an ‘appropriate anion’ that favourably combines with many aromatic amines and alkaloids which may ultimately be extracted from the corresponding aqueous solutions into chloroform as their respective chlorides*.

4. EFFECT OF SYNERGISTIC EXTRACTION

Synergism: It may be defined as ‘the process whereby two different reagents when employed together are capable of extracting a metal ion with a distinct and marked efficiency, in comparison to a condition when the same two reagents are used individually’.

Example: (i) : Complexation of Mn²⁺ with dithizone and pyridine:

It has been observed that the complex formed by Mn²⁺ with dithizone alone is of no practical analytical utility because of the fact that it undergoes decomposition very quickly. However, the addition of a base, such as pyridine into the Mn²⁺ plus dithizone complex yields a red-complex, which is fairly stable to oxidation and light; and, therefore, forms the basis for a very sensitive photometric method employed in estimating trace amounts of Mn²⁺.

Equation: Following is the chemical reaction of the above complex formation:

(ii) Complexation of Ni²⁺ with dithizone and 1, 10-phenanthroline:

Noticeably, the reaction of Ni²⁺ with dithizone is quite slow and sluggish. Nevertheless, this slow reaction is significantly accelerated by the addition of nitrogen-containing bases like 1, 10-phenanthroline. The resulting complex may be represented by the following equation:
**Equation:**

\[
\text{Ni}^{2+} + 2\text{S} = C
\]

![Diagram of chemical reactions involving nickel, dithizone, and 1,10-phenanthroline.](image)

It is the basis of a very sensitive synergistic extraction photometric procedure for trace amounts of \(\text{Ni}^{2+}\).

---

**RADIOIMMUNOASSAY**

**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 endocrinology 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 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

- Non-specificity of the technique,
- Non-sensitivity of the method,
- Involvement of the processes of extraction, purification and concentration of the specimen under investigation,
- Heat treatment of the specimen resulted invariably in degradation and destruction of the substances, and
- 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, phar-macologist, 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 concen-trations 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, anti-bodies, 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 mean-ingful contributions by Landon and Moffatt**** (1976) proved beyond any reasonable doubt the efficacy of RIA in the quantification of a host of pharmaceutical substances.

Radioimmunoassay: Theory

1. Hapten Determinants and Purity: The Key to Immunological Specificity 2. Importance of Antigenic Determinants 3. Analysis by Competitive Antibody Binding or Isotopically Labelled Compounds

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 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*.

1. HAPTON 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 conju-gation to macromolecule the loss of even one functional group can turn out to be critical.

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.

(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.

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.
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 (unla-belled). 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

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:

\[
\frac{(A - P + A* - P)}{\text{Total} (A + A*)} \quad \text{or} \quad \frac{A* - P}{\text{Total} A*} \quad \text{and} \quad \frac{A - P}{\text{Total} A}
\]

where, \((A* - P) = \text{Antibody labelled antigen-complex, and}

\((A - P) = \text{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:
If a further 100 molecules of A are added at this stage, the percentage bound shall become:

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

or

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

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.](image)

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.
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.

**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:
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.

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.

2.1. Gamma Counters

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

2.2. Scintillation Counters

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

First and foremost, radioimmunoassays were universally based on the $^3\text{H}$ or $^{14}\text{C}$ 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 $^{124}\text{I}$, $^{125}\text{I}$, or $^{131}\text{I}$ is now being increasingly utilized wherever such labelling is practically feasible.

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 some 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.
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 — Narcotic analgesic, antitussive and antipyretic, Hydrocodone 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 — B1-Adrenoreceptor blocker used in hypertension and

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

1. RADIOIMMUNOASSAY OF MORPHINE

Synthesis of Immunogen: Morphine is first converted to 3-α-carboxymethylmorphine by reacting the free base with sodium-p-chloroacetate in absolute ethanol

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) carbiidiimide 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 \(^{3}\text{H}\) 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, The tube which contained radioactive dihydromorphine and antiserum but no unlabelled morphine, served as a measure of maximum antibody-bound radioactivity,

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

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

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 proprietary 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.
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.

RIA is carried out using morphine-6-antiserum and tritiated dihydromorphine (commercially avail-able). 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

(i) **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.

**RIA-Procedure** : The different steps to be followed are stated below, namely :
a) Various dilutions of unknown plasma, morphine-6-antiserum, $^3$H-dihydromorphone are prepared afresh,

b) 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,

c) 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,

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

e) 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 scintillation fluid,

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

g) 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

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

3. RADIOIMMUNOASSAY OF CLONAZEPAM

**Clonazepam** 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*

* [Chemical Structure Image]
**Synthesis of Immunogen** : The 3-hemisuccinylloxy 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 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)-clonazepam 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 $^3\text{H}$-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 $^3\text{H}$-counts is employed for the percentage of binding.

4. RADIOIMMUNOASSAY OF FLURAZEPAM IN HUMAN PLASMA

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

\[
\text{Flurazepam}
\]

**Synthesis of Immunogen (Hapten)**: 3-Hydroxy flurazepam is refluxed with succinic anhydride in dichloromethane containing triethylamine to produce the desired hapten, 3-hemisuccinylxyloxy-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-hemisuccinylxyloxyflurazepam, 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:

- A calibration curve is generated by adding $^3\text{H}$-Flurazepam in 0.1 ml of buffer containing 0.03-0.2 ng range of flurazepam in buffer,
- Following preparation of the standards, duplicate portions of the reconstituted unknown flurazepam fractions are added to tubes containing $^3\text{H}$-Flurazepam,
- Diluted antiserum is added to all tubes except the non-specific-binding control specimen to which buffer is added,
- The contents of each tube is mixed gently on a Vortex Mixer and allowed to stand at room temperature,
Following incubation, the antibody-bound radio ligand is separated from the unbound fraction by precipitation with saturated ammonium sulphate.

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

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-desethyl metabolites 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>
<thead>
<tr>
<th>S.No.</th>
<th>Compound</th>
<th>Cross-Reactivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Flurazepam</td>
<td>100</td>
</tr>
<tr>
<td>2.</td>
<td>3-Hexamethoxyfluorazepam</td>
<td>—</td>
</tr>
<tr>
<td>3.</td>
<td>3-Hydroxyfluorazepam</td>
<td>—</td>
</tr>
<tr>
<td>4.</td>
<td>Monodesethylfluorazepam</td>
<td>17.0</td>
</tr>
<tr>
<td>5.</td>
<td>Didesethylfluorazepam</td>
<td>3.5</td>
</tr>
<tr>
<td>6.</td>
<td>N-1-Hydroxyethylfluorazepam</td>
<td>1.0</td>
</tr>
<tr>
<td>7.</td>
<td>N-1-Desalkylfluorazepam</td>
<td>1.0</td>
</tr>
</tbody>
</table>

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.

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.
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 °C 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 lyophilized 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 up to 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-14C,

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-14C 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*

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Compound</th>
<th>Cross-Reactivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Chlordiazepoxide</td>
<td>100</td>
</tr>
<tr>
<td>2.</td>
<td>N-Desmethylichlordiazepoxide</td>
<td>5</td>
</tr>
<tr>
<td>3.</td>
<td>Demoxepam</td>
<td>&lt;1</td>
</tr>
<tr>
<td>4.</td>
<td>N-Desmethyldiazepam</td>
<td>&lt;1</td>
</tr>
<tr>
<td>5.</td>
<td>Diazepam</td>
<td>&lt;1</td>
</tr>
<tr>
<td>6.</td>
<td>Clonazepam</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

From Table 32.2 it is evident that the highest cross-reaction is 5% with N-desmethylichlordiazepoxide 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.

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 up to 5 ng without indulging in any type of extraction, filtration or evaporation as required for other conventional analytical methods**.

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-nitrophenyloxy carbonylisopropyl) barbituric acid (II) by the interaction of the base with p-nitrophenol in N, N-dimethylformamide (DMF) as shown below:

![Chemical structure](image)
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 immunized 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.
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 molecule](image)

**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:

- 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,

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

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

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

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.

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.

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.

The incubation is done overnight.

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

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 calculated percent-age inhibition value as depicted in figure 32.5 below:

![Figure 32.5: Standard Curve (Displacement Curve) of Tritiated Flunisolide.](image-url)