Name of Unit	Radioimmuno Assay and Extraction Techniques
Course/Subject Name	Advanced Instrumentation Techniques
Course/Subject Code	BP811ET
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Learning Outcome of Module-IV

LO	**Learning Outcome (LO)**	**Course Outcome Code**
LO1	To understand the basic concept of Radioimmuno Assay.	BP811.1, BP811.5
LO2	To know the importance, various components, Principle, different methods.	BP811.5
LO3	To understand limitation and applications of Radioimmuno assay	BP811.5
LO4	To understand the general principle and procedure involved in the solid phase extraction.	BP811.1, BP811.5
LO5	To understand the general principle and procedure involved in the liquid- liquid extraction.	BP811.1, BP811.5

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RADIOIMMUNOASSAY

INTRODUCTION

The introduction of radioimmunoassay (RIA) and its subsequent development as a possible versatile tool in wide spheresof scienceoccurred empirically to the initiaton. 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 layer chromatography (TLC), gas liquidchromatography (GLC), spectrofluometry (SPF) and ordinary radiolabelling assay. The above methods, undoubtedly, have certain advantages to their credit; however, the disadvantagesoutnumbered 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 method for biological fluids.

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.

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.

Importance of Antigenic Determinants

The functional groups of the hapten should remain unblocked in the conjugate molecule, 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, 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.

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.

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.

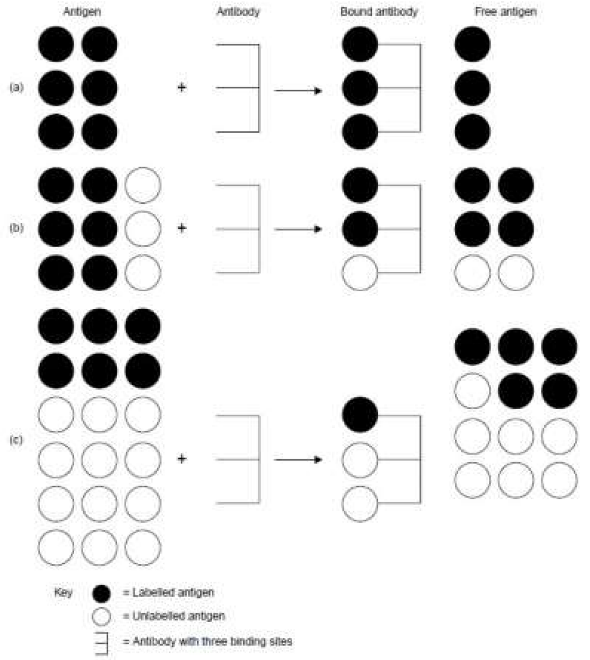


Figure 32.1 : Schematic Illustration of Competitive Binding in Ideal Radioimmunoassay.

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

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

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,

The antigen and antibody usually react in the ratio 1:1, and 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:

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:

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: 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 32.3, 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:

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, 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 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: Centrifuge, and Radioactive Counters.

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 formedduly.

RADIOACTIVE COUNTERS

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

Gamma Counters, and

Scintillation Counters.

Gamma Counters

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

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 tech-nique, 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.

METHODOLOGY OF THE ASSAY

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

Mix a fixed volume (fixed concentration) of antiserum containing the specific antibody with a constant amount of radiolabelled antigen, Incubate it for come specified duration at an appropriate temperature, usually + 4 °C, A definite volume of the sample containing the hapten to be measured is added to the reaction test-tube, The antibody reacts with both the radioactive and unlabelled hapten forming an antibody-radiolabelled antigen and antibody-unlabelled antigen complexes, 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, The radioactivity falls because the unlabelled antigen dilutes it i.e., reducing the number of la-belled hapten combining with the antibody, 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 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.

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, 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, 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, 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 inter-est. 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) INPHARMACEUTICAL 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, Hydrocodone: Narcotic analgesic, antitussive and antipyretic,

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.

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 resonably. 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 ARRARappropriate 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 theirusual estimation either by chromatographic techniques or by atomic-absorption methods.

Liquid-Liquid Extraction: Theory

1. Error Due to the Volume Change
2. 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 phase 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: Where,

$$K_p = \frac{[A]_a}{[A]_b} = \frac{\text{Concentration of solute in solvent ‘}a\text{’}}{\text{Concentration of solute in solvent ‘}b\text{’}} \qquad ...(a)$$

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:

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

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 Kp 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:

$$K_p = \frac{S_1}{S_2} \qquad ....(b)$$

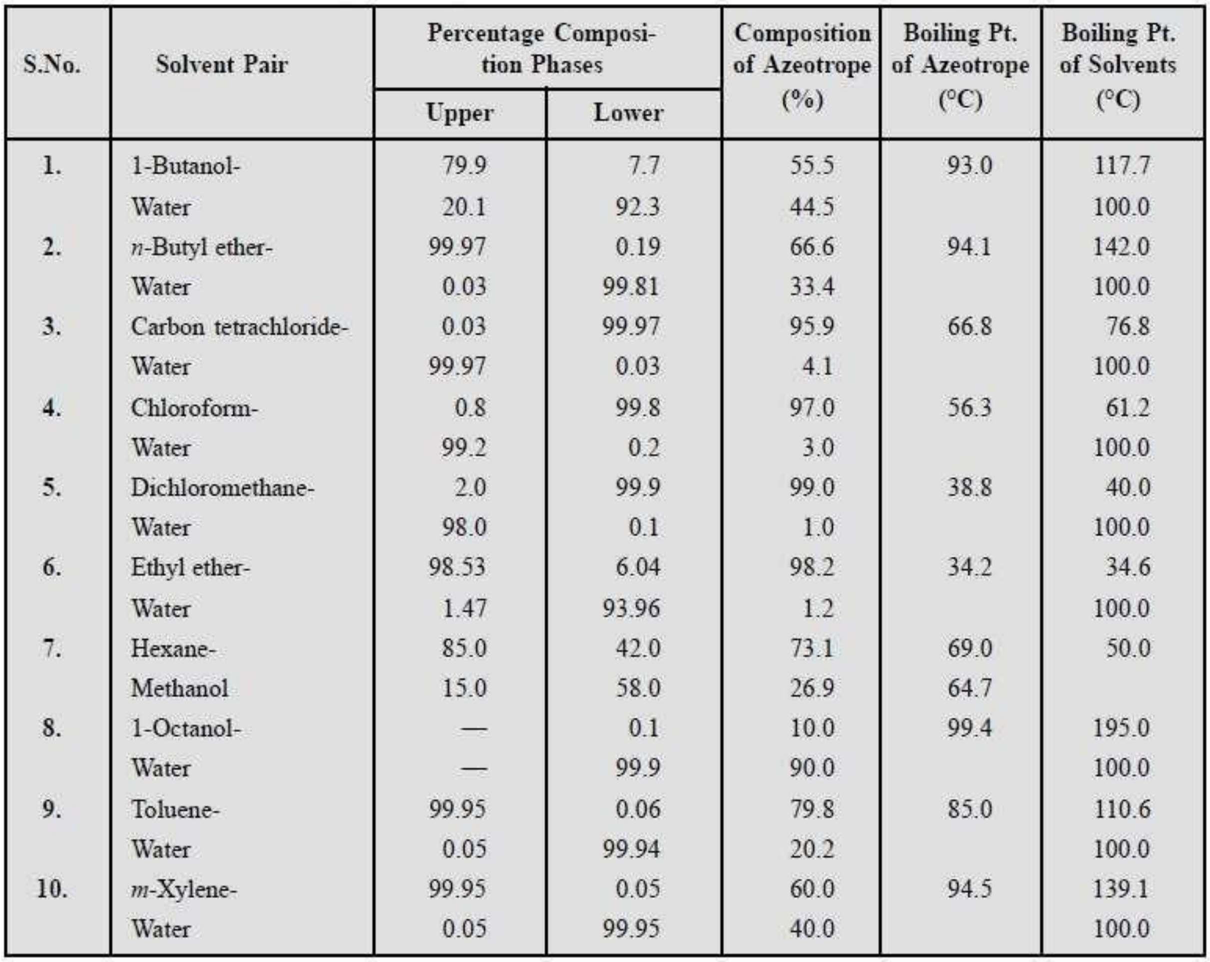
where, S_1 = Solubility of substance A in solvent ‘*a*’, and S_2 = Solubility of substance A in solvent ‘*b*’.

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.

Table 27.1 : Physical Data on Binary Solvent Systems*



S.No.	Solvent Pair	Percentage Composition Phases		Composition of Azeotrope (%)	Boiling Pt. of Azeotrope (°C)	Boiling Pt. of Solvents (°C)
		Upper	Lower			
1.	1-Butanol-	79.9	7.7	55.5	93.0	117.7
	Water	20.1	92.3	44.5		100.0
2.	*n*-Butyl ether-	99.97	0.19	66.6	94.1	142.0
	Water	0.03	99.81	33.4		100.0
3.	Carbon tetrachloride-	0.03	99.97	95.9	66.8	76.8
	Water	99.97	0.03	4.1		100.0
4.	Chloroform-	0.8	99.8	97.0	56.3	61.2
	Water	99.2	0.2	3.0		100.0
5.	Dichloromethane-	2.0	99.9	99.0	38.8	40.0
	Water	98.0	0.1	1.0		100.0
6.	Ethyl ether-	98.53	6.04	98.2	34.2	34.6
	Water	1.47	93.96	1.2		100.0
7.	Hexane-	85.0	42.0	73.1	69.0	50.0
	Methanol	15.0	58.0	26.9	64.7	
8.	1-Octanol-	—	0.1	10.0	99.4	195.0
	Water	—	99.9	90.0		100.0
9.	Toluene-	99.95	0.06	79.8	85.0	110.6
	Water	0.05	99.94	20.2		100.0
10.	*m*-Xylene-	99.95	0.05	60.0	94.5	139.1
	Water	0.05	99.95	40.0		100.0

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

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

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

or
$$y/x = \left(\frac{V_1}{V_2}K_p + 1\right)^{-1} = f$$

In liquid-liquid extractions the following *two* aspects are very crucial and important, namely:

- Error due to the Volume Change, and
- Effectiveness of an Extraction.

These *two* aspects shall be discussed briefly at this juncture.

ERROR DUE TO THE VOLUME CHANGE

In a situation where in 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: Measure the volume of the phase employed for the analysis and incorporate this volume in the calculations, Separate the phase quantitatively and subsequently dilute to a known volume,

Separate the phase quantitatively and make use of the entire volume in the remaining steps of the ongoing analysis, and 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.

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 V2 of Solvent ‘b’. Now, this particular sample undergoes extraction with a volume V1 of Solvent ‘a’ and subsequently ‘y’ moles of com-pound are left in V2 at equilibrium.

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

$$K_p = \frac{M_1}{M_2} = \left(\frac{x-y}{V_1}\right) \Big/ \left(\frac{y}{V_2}\right) \qquad \ldots(c)$$

After simplifying and rearranging :

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 ‘*n*’ extraction may be given by the following expression:

$$fn = \left(\frac{V_1}{V_2} K_p + 1\right)^{-n} \qquad . \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (e)$$

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 ofthe analyte left in the sample after a single extraction:

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

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’.

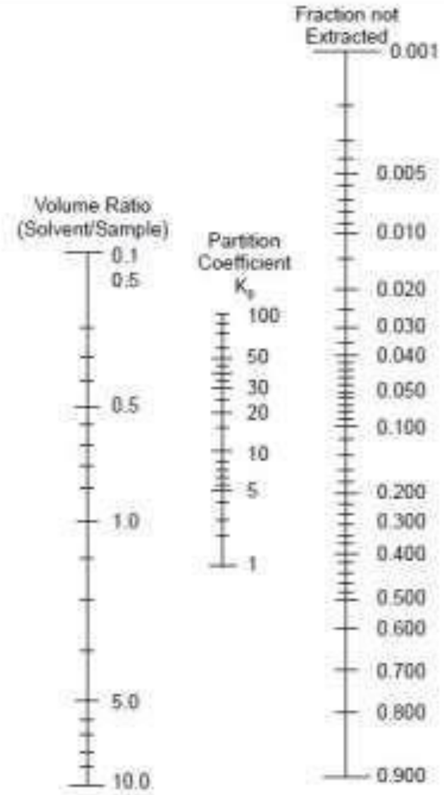


Figure 27.1 : A nomogram to calculate the 'fraction not extracted' after a single step liquid-liquid extraction process.

FACTORS INFLUENCING SOLVENT EXTRACTION

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

Effect of temperature and inert solutes,

Effect of pH on extraction,

Effect of ion-pair formation, and

Effect of synergistic extraction.

These factors shall be discussed briefly below:

EFFECT OF TEMPERATURE AND INERT SOLUTES

The physical as well as chemical interactions of a solute are 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. Bysubstituting 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.

EFFECT OF pH ON EXTRACTION

Generally, it has been found that the organic acids and bases do exist in aqueous solution as equilib-rium 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 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 :

$$E = \text{Extraction Coefficient} = \frac{\Sigma[\mathrm{Si}]_2}{\Sigma[\mathrm{Si}]_1} \qquad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\text{(i)}$$

where, $\Sigma[Si]_2$ = The sum total of all forms of the compound in Phase-‘2’, and $\Sigma[Si]_1$ = 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 theextrac-tion coefficient for the system :

$$A + H \rightleftharpoons AH \quad \text{or} \quad K = \frac{[AH]_1}{[A]_1\,[H]_1}$$ ....(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 $$= \frac{[A]_1\ \{K_p.A + K_p.AH[AH]_1 / [A]_1\}}{[A]_1\ \{1 + [AH]_1 / [A]_1\}}$$

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

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 conjugateacid),

Eq. (*v*) may be expressed as :

$$E = K_p,\ A\ \frac{1}{1 + K[H]_1} = K_p,\ A\ \frac{[A]}{[A] + [AH]} \qquad ...(vi)$$

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

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

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

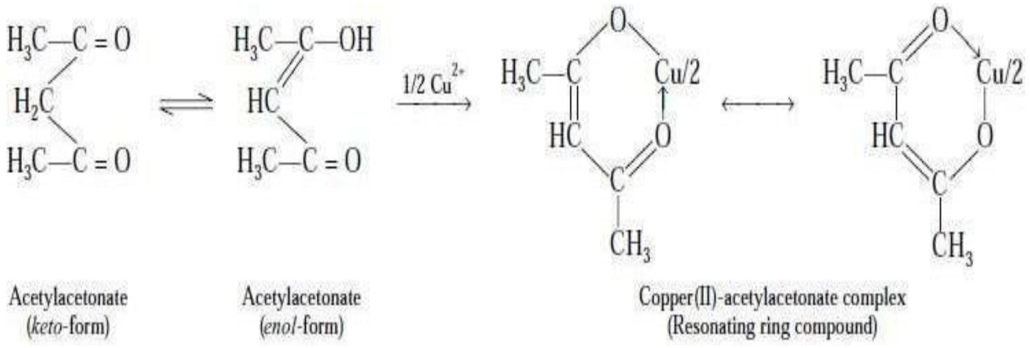
Forcibly shifting the ‘equilibrium’ toward the extractable species by adjusting the pH helps to minimise the effect of the equilibrium thereby rendering E almost equal to K_p, A.

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.

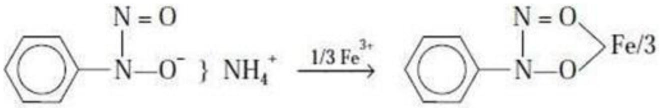
EFFECT OF ION-PAIR FORMATION

Ion-pair formation needs its due recognition because it very often gives rise to unexpected extrac-tions. 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 compo-nent 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.

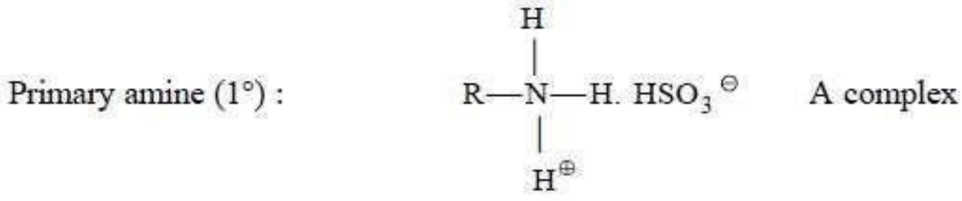
A few typical examples shall be discussed here to explain the chelate-formation: Example 1: Cu^{2+} with ‘acetylacetonate’ forms a fairly stable ring compound:



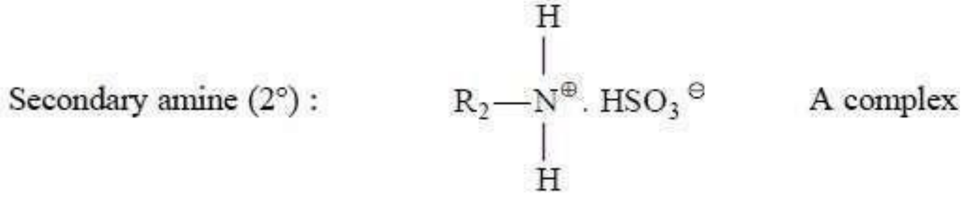
***Example 2* :** Iron (III) 'cupferrate' gives rise to a stable ring compounds as shown below :



***Example 3* :** Sulphonic acids rapidly pair with a plethora of 'protonated amines' to form an easilyextractable complex



Primary amine (1°) : $R-\overset{H}{\underset{H^{\oplus}}{N}}-H.\ HSO_3^{\ominus}$ A complex



Secondary amine (2°) : $R_2-\overset{H}{\underset{H}{N^{\oplus}}}.\ HSO_3^{\ominus}$ A complex

***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*.

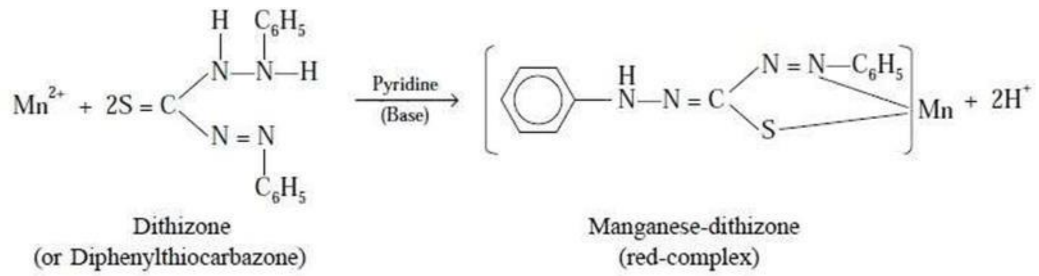
EFFECT OF SYNERGISTIC EXTRACTION

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 Mn^{2+} with dithizone and pyridine :

It has been observed that the complex formed by Mn^{2+} with dithizone alone is of no practical analyti-cal utility because of the fact that it undergoes decomposition very quickly. However, the addition of a base, such as : pyridine into the Mn^{2+} 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 Mn^{2+}.

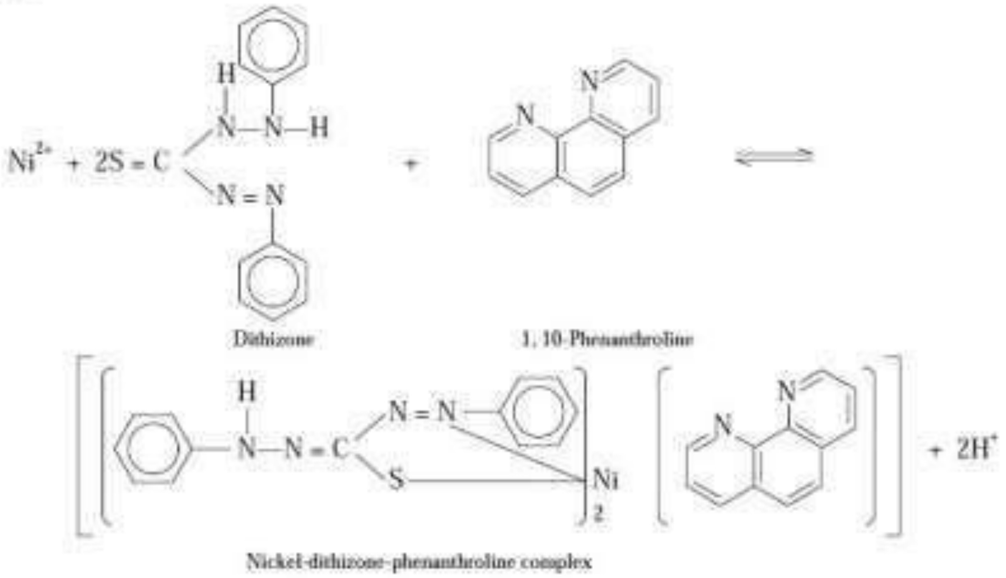
Equation : Following is the chemical reaction of the above complex formation :



(ii) Complexation of Ni^{2+} with dithizone and 1, 10-phenanthrolone:

Noticeably, the reaction of Ni^{2+} 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 :



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

IMPORTANT QUESTIONS

Long answer type Questions (10 Marks)

1. Explain in details about principle, process and separation techniques of RIA
2. Explain the instrumentation of RIA
3. Give application of extraction methods
4. Explain in detail continuous liquid-liquid extraction methods
5. Write a detail about the factors affecting the liquid-liquid extraction.

Short answer type questions (5Marks)

1. What is decoction? Explain with an example
2. Explain about Soxhlet apparatus
3. Write a short note on Maceration
4. Briefly write about percolation
5. Enlist the various Liquid-liquid extraction methods
6. Give advantages and disadvantages of RIA
7. Write applications of RIA.
8. Give theory and principle of RIA
9. Draw a well labeled diagram of RIA
10. Enlist the various methods of solid liquid extraction methods

Very Short answer type questions (2 Marks)

1. Define Partition Coefficient
2. Principle of Liquid-Liquid extraction
3. Name the methods for the extraction of solids
4. Enlist the factors affecting the value of partition coefficient
5. Define Decoction
6. Define percolation
7. What is Hapten?
8. Give examples of Radiolabeled antigen
9. Enlist the various counters used in RIA
10. Define Radioimmunoassay.