Role of Isotopic Dilution Thechnique in Analytical and Clinical Assay of Drugs

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Isotopic Dilution Analysis (IDA) technique in the field of advanced analysis of pharmaceutical products has been proved to be a vital tool for the assessment of drug contents. It is not only confined in the field of pharmaceutical analysis, but is also applicable in inorganic, organic, biochemistry and in some special procedure like determination of volume, dissolution constant, capacities of ion exchangers etc.

The principle of IDA is based on the fact that if a known amount of a radiotracer is mixed with an unknown amount of the same unlabelled compound the extent of dilution could be measured in terms of reduction of the specific activity of the original radiotracers.

The following equation may be applied for the determination of the amount of substance (Wx) present in an unknown sample.

$$Wx = Wo \left(\frac{AoW_2}{A_2 Wo} - 1 \right) \quad \text{(i)}$$

$$\text{or, } Wx = Wo\,(So/S_2 - 1) \quad \text{(ii)}$$

Where, Wo = Wt. of a radioactive nucleide of known radioactivity Ao; W_2 = the weight of isolated substance; Ao = radioactivity of known radioactive nucleide; A_2 = radioactivity of isolated substance; So = A_0/W_0, initial specific activity; $S_2 = A_2/W_2$, specific activity of isolated substance.

The error arising from the uncertainity in A_0 and A_2 may be calculated from the following equation.

$$Er = \frac{\Delta Wx}{Wx} = \left(\frac{1 + Wo}{Wx} \right) \left[\left(\frac{\Delta So}{So} \right)^2 + \left(\frac{\Delta S_2}{S_2} \right)^2 \right]^{1/2} \quad \text{(iii)}$$

The influence in error due to the instability of the whole measuring apparatus is also neglected besides uncertainity in W_0 and W_2. As an example, the error of the measuring apparatus NZB315 for measuring ^{14}C is guaranteed by the manufacturer to be less than 0.5%[17].

IDA technique has been proved to be a vital method for the assay and determination of many substances, where no other appropriate method is available. It was first used by Heavesy and Hobbie for the determination of lead in minerals. Much of the work has been devoted to the determination of proteins and aminoacids, steroids, vitamins, antibiotics, insecticides etc.

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Penicillin :

Estimation of penicillin by IDA method was reviewed[7,8]. Previously the methods used for the assay of penicillin has been the relatively inaccurate biological procedures. In this method, benzyl penicillin (Penicillin G) labelled with ^{35}S was used which had been prepared by biological synthesis in the presence of $K_2{}^{35}SO_4$. The labelled penicillin is added to the test solution and a portion of the sample is separated as N-ethyl-piperidine salt. If the substance contains less than 10,000 units of penicillin, the error of the determination was 5.9%.

Steroids :

A review article on steroid analysis[3,4,6,11,21,22] include the estimation of aldosterone, testosterone, cortisol and stigmasterol either by isotope derivation dilution or by double isotope derivation dilution[3,11]. A scheme for their determination by IDD method is[34] :

Unlabelled steroid(s) in sample
↓
extraction and concentration
↓
reaction with ^{14}C-acetic anhydride ← non-radioactive acetate of interest added
↓
isotopic derivative acetate
↓
purification by chromatography
↓
elution and concentration→
two aliquots : (a) chemicalassay to determine the amount of substance.
(b) ^{14}C counting to determine the amount of radioactivity and to calulate the specific activity of the acetate derivative.

As little as 20(μg) of unknown substance can be detected in this way. In double isotope derivation dilution two different isotopes are used. These isotopes are ^{3}H acetic anhydride and ^{14}C-labelled steroid acetate of interest. Determination of testosterone in peripheral plasma is based on a double labelling technique with the use of ^{3}H testosterone and ^{35}S-thiosemicarbazide.

Cyanocobalamin :

Estimation of cyanocobalamin in a pharmaceutical product, or in liver was reviewed. The determination of vitamin B_{12} by IDA technique has been elaborated, by Rosenblum *et al.*[26]. Vitamin B_{12} has been determined in fermentation liquors, in living tissues, in animal faeces etc. by simple or reverse IDA[5,16,25,31]. IDA method of analysis of vitamin B_{12} has been appended to the U. S. Pharmacopoeia[32].

Most of IDA of vitamin B_{12} is carried out by labelling the cyanide group of vitamin B_{12} with ^{14}C, introduced by chemical exchange. Vitamin B_{12} of high specific activity, labelled with cobalt, can be prepared by fermentation in the presence of low concentrations (0.1 ppm) of radioactive cobalt.[5] Most frequently, labelling is carried out by ^{60}Co ($t_{1/2}$ = 5.2 yrs) and ^{58}Co ($t_{1/2}$ = 72 days).

Assay of vitamin B_{12} in pharmaceutical preparation by isotope dilution analysis method is represented in table-1[25].

Tabe 1 : Assay of Vitamin B_{12} in pharmaceutical product by IDA.

Types of sample tested	Labelled claim	Result of assay			
		IDA	U. S. P. microbial method	Spectro photometry	Chick growth
Cobalamine concentrate	950-135μg/g	1076	1117	—	—
Same + 200μg of red pigments	same + 200μg/g	1076	1186	—	—
Liver injection	50μg/ml	51.8	53.7	—	—
Same + red pigments	same + 16μg/ml	52.7	59.9	—	—

IDA of vitamin B_{12} using ^{60}Co was described by Konecny and Tologyessy[16]. A method for the diagnosis of pernicious anaemia was also described by Tabern and Storey[31], in which Vit. B_{12} labelled with radioactive ^{60}Co was used.

Proteins and Amino Acids :

It has been experienced to be extremely difficult to analyse the amino acids formed during the hydrolysis of proteins, whether effected by enzymes or chemical reagents.[24] In such determinations the IDA method was found to be very useful. The total amount of amino acid in the assay preparation may be measured after a known quantity of the desired substance, enriched with respect to its content of stable nitrogen isotope of mass 15 (^{15}N), is added and mixture resolved.

The radioactive added amino acid was isolated (need not be quantative) and its specific activity, determined by mass spectrometry, indicates the amount of amino acid recovered. From this the concentration of amino acid in the original preparation is calculated.

Shemin and Foster[27] reported that the estimation of amino acids in protein hydrolysates can be made with an expected accuracy of 1 to 2%.

Other methods of determination of amino acids involves the preparation of radioactive derivatives of the single amino acid in the test solution by adding a radioactive reagent. p-^{131}I-phenylsulphonylchloride is usually used as the radioactive reagent. A large amount of non-radioactive pipsyl derivative of the desired single amino acid is then added as a carrier (single indicator method)[18] and the active and inactive derivatives of this amino acid are separated by paper chromatography of recrystallization. This permits calculation of the original amount of active derivative, and consequently, the amount of amino acid present in the hydrolysate or mixture.

Two indicator method ^{131}I and ^{35}S for the estimation of amino acid in the mixture was described by Keston, Udenfriend and Levy[14,15]. Glutamic acid, serine, glycine and alanine were determined using this method. In the hydrolysate of natural milk[14], ^{14}C—amino acids can also be used in place of ^{35}S—pipsyl amino acids.

Biological Fluids :

The clinical application of IDA technique is elaborated by its use in the measure-

ment of blood volume, extracellular fluid volume, erythrocyte volume etc. The basic principle is the same in all the determinations, with the variation of different radioactive species.

Double isotope labelling in toxicological analysis of strychnine in blood was reviewed. It has been experienced that strychnine can be analysed more accurately by this method than by more conventional procedure[35] by using ^{14}C and ^{3}H—labelled strychnine methiodide.

The more popular procedure for the determination of blood volume uses radioiodinated human serum albumin injected iv; 10 minutes after injection, a time sufficient to allow adequate mixing of the labelled albumin in the intravascular pool, yet not long enough for metabolic activity, a blood sample is withdrawn. The blood volume is calculated from the measured decrease in radioactivity of the injected sample upon its dilution by the blood[23].

Rowland, Freeman and Fleming[36] described a method for the measurement of blood volume in haemorrhagic hypotension using a double tracer technique. The method is used to determine blood volume before and after haemorrhage in animals. Direct evidence is offered of sequestration of part of the blood volume from the general circulation in state of extreme hypotension.

A review in the determination of cardiac output in human subjects describing the method of determination proved to be a very useful technique. Veall *et al.*[38] developed a technique for cardiac output using the radioactive isotope ^{131}I. A known volume of about 2-3 ml of ^{131}I-labelled serum albumin containing about 200μc of ^{131}I is injected rapidly into the left antecubital vein and chart recorder switched on. After reaching an equilibrium counting rate, a venous blood sample is withdrawn from the other arm for calibration purpose. The activity of blood sample is compared with that of a 1/500 dilution of the injected materials. The cardiac output may be calculated by applying the following equation.

$$F = Q / a \int_{0}^{\infty} N dt$$

Where, N = instaneous counting; Q = known quantity of substance injected; a = calibration factor.

Further reports deal with the determination of Caffeine[18], D-glucose and gentibiose[28], insulin[10], thiamine[9],[19], sulphapheenazole[30], giberallic acid[1], griseofulvin[2], Vitamin D[32], lactonitriles and various other substances by IDA.

Estimation of total body water in mammals by Deuterium dilution was recently reviewed[29]. Stewart and Hulston[29] pointed out that the standard technique previously employed for the determination of total body water by administrating D_2O has led to the total body water being overestimated by about 10 precent.

It thus appears that the isotopic dilution technique is finding increasing use in pharmaceutical field for their accuracy, sensitivity and determination of trace amount of substances in body fluid where other techniques fail.

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