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Review Article

A Review on Tracer Technique and Its Applications in Synthesis of Secondary Metabolites in Pharmacognosy

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ABSTRACT

Many classical biochemical methods are available to study the primary metabolic pathway such as krebs cycle, glycolysis and HMP shunt pathway in living organisms. Number of advanced techniques has been developed to perform the bio synthetic study. The present review deals with the tracer technique and its applications in synthesis of secondary metabolites in pharmacognosy. This technique involves the stable (1H2,6C13,7N15,8O18) and unstable (1H1,6C14) radioactive isotopes. These isotopes are in corporate into presumed plant precursors with which they form a chemical bond and can be easily detected by various methods like liquid scintillation, giger muller and auto radiography techniques. These isotopes are regarded as radioactive markers in biosynthetic studies.

1. Introduction

A radioactive tracer, radiotracer, or radioactive tag is a chemical compound in which one or more atoms have been replaced by a radionuclide so that, due to its radioactive decay, it can be used to investigate the mechanism of chemical reactions by following the path that the radioisotope takes from the reactants to the products. Radio labeling or radio monitoring is therefore the radioactive component of an isotope tag.

Hydrogen, carbon, phosphorus, sulfur and iodine radioisotopes have been used extensively to map the course of biochemical reactions. A radioactive tracer may also be used to track the distribution of a material within a natural system, such as a cell or tissue [1], or as a stream tracer to track fluid flow. Radioactive tracers are also used to determine the location of fractures caused by fractures Produced by hydraulic fracturing in the production of natural gas. [2] Radioactive tracers form the basis of a wide range for imaging devices, such as PET scans, SPECT scans and technetium scans. Radiocarbon dating uses the naturally occurring carbon-14 isotope as an isotope tag.

The isotopes of the chemical element vary only in weight. For example, hydrogen isotopes can be written as ^{1}H, ^{2}H, and ^{3}H, the mass number being superseded to the left. When the atomic nucleus of the isotope becomes unstable, the substances comprising the isotope are radioactive. Tritium is an example of a radioactive isotope.

The idea behind the use of radioactive tracers is that an atom in a chemical compound is replaced by another atom with the same chemical element. Nevertheless, the substituting atom is a radioactive isotope. This method is often referred to as nuclear marking. The effectiveness of this method is due to the fact that radioactive decay is much more energetic than chemical reactions. Radioactive isotopes can therefore be present at low concentrations and measured by sensitive radiation detectors such as Geiger counters or scintillation counters. George de Hevesy received the 1943 Nobel Prize in Chemistry for his work on the use of isotopes as tracers in the study of chemical processes.

There are two main ways in which radioactive tracers are used.

- When a labeled chemical compound undergoes chemical reactions, one or more of the components will have a radioactive tag. Analysis of what

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happens to the radioactive isotope provides detailed information on the chemical reaction process.

- A radioactive compound is inserted into the living organism and the radioisotope provides a means of creating a picture showing the way in which the compound and its reaction products are dispersed around the organism.

2. Tracer Isotopes

Hydrogen: Tritium is formed by neutron irradiation of

$$6Li + n \rightarrow 4He + 3H$$

Tritium has a half-life of 4,500±8 days (approximately 12,32 years)[4] and decays by beta decay. The average energy generated by the electrons is 5.7 keV. Because the released electrons have relatively low energy, the performance of scintillation detection is rather small. Nevertheless, hydrogen atoms are present in all of them Tritium is often used as a tracer in biochemical research.

Carbon: ^{11}C is decayed by positron emission with a half-life of ca. 20 min. ^{11}C is one of the isotopes often used in positron emission tomography.[3] ^{14}C decays by beta decay, with a half-life of 5730 y. It is continually generated in the upper atmosphere of the earth so that it exists at a trace rate in the environment. Nevertheless, it is not feasible to use the naturally occurring ^{14}C for tracer studies. Alternatively, neutron irradiation of the isotope ^{13}C occurs naturally in coal at around 1.1 per cent. ^{14}C has been extensively used to track the progress of organic molecules across metabolic pathways [5].

Nitrogen: ^{13}N decays by positron emission at a half-life of 9.97 min. It is generated by a nuclear reaction of

$$^{1}H + {}^{16}O \rightarrow {}^{13}N + {}^{4}He$$

^{13}N is used in positron emission tomography (PET scan).

Iodine: ^{123}I is formed by ^{124}Xe proton irradiation. The isotope formed by caesium is unstable and decays to ^{123}I. The isotope is usually supplied as the iodide and hypoiodate in dilute sodium hydroxide solution, at high isotopic purity, [6] ^{123}I has also been produced at Oak Ridge National Laboratories by proton bombardment of ^{123}Te. [7] ^{123}I decays by electron capture at a half-life of 13.22 hours. The emitted 159 keV gamma ray is used in single photon emission computed tomography (SPECT) applications. A 127 keV gamma ray is also being emitted. ^{125}I is frequently used in radio immunoassays due to its relatively long half-life (59 days) and ability to detect gamma counters with high sensitivity [8]. ^{129}I is present in the environment as a result of nuclear weapons testing in the atmosphere. It was also the product of the Chernobyl and Fukushima accidents. ^{129}I decays with a half-life of 15.7 million years and low-energy beta and gamma emissions. This is not used as a tracer, although its existence in living organisms, including human beings, can be characterized by gamma rays.

Other isotopes: Many other isotopes have been used in advanced radio pharmacology research. The most commonly used is ^{67}Ga for gallium scanning. ^{67}Ga is used because, like 99mTc, it is a gamma-ray emitter and different ligands can be bound to the Ga^{3+} ion, forming a coordination complex that may have selective affinity to specific sites in the human body.

Living plants are known to be biosynthetic laboratories for the processing of both primary and secondary metabolites. Different intermediates and steps are involved in biosynthetic processes in plants that can be studied using the following techniques:

- *Isolated Organ / Tissues:* This approach is focused on the use of isolated sections of plants (e.g. stem roots, etc.). This method is useful for the determination of the site of the synthesis of a particular compound. Ex.: Roots and leaves for the study of Nicotiana and Datura, Petal disk for the study of rose oil, Tropane alkaloids developed at the root of the Solanaceae family.
- *Grafting methods:* This process is used for the study of the production of alkaloids by Grafted Plants. Ex.: Tomato Scions grafted on Datura accumulate alkaloids, while Datura Scion grafted on tomatoes contained a very small amount of alkaloids. This suggests that the main site for the formation of Datura alkaloids is Root.
- *Use of mutant strains:* This approach is used to generate mutant strains of microorganisms that lack certain enzymes. Ex.: Gibberella mutant is used to manufacture isoprenoid compounds; Lactobacillus acidophilus is used for Mevoloinic Acid Pathway of Isoprenoid compound synthesis.
- *Tracer method:* This technique uses a marked compound to find / trace various intermediates and different phases in the Biosynthetic Pathway 3

Here introduces the paper, and put a nomenclature if necessary, in a box with the same font size as the rest of the paper. The paragraphs continue from here and are only separated by headings, subheadings, images and formulae. The section headings are arranged by numbers, bold and 9.5 pt. Here follow further instructions for authors.

3. Tracer Technique

It can be described as a technique that uses a marked compound to identify or trace specific intermediates and different steps in biosynthetic pathways in plants at a given rate and time. This technique often uses marked compounds that, when introduced into the plant system become part of the general metabolic pool and undergoes reactions. Associated with that system of plants. This method was used specifically for the determination of primary metabolites and secondary metabolites in biosynthetic pathways.

Metabolic products of plants: A living plant can produce two kinds of useful metabolites by absorbing food, water, sunlight and minerals.

Primary metabolites: These compounds are abundant in nature and are found in various forms in all types of species. They are important for the normal growth and development of the organism.

Secondary metabolites: These are chemical compounds biosynthetically derived from principal metabolites such as carbohydrates. fats, proteins, mineral nutrients and vitamins etc. E.g. Alkaloids, Terpenoids, Glycosides, Flavanoids, Tannins and Coumarins

3.1. Significance of Tracer Technique

• Applicable for living systems. Wide ranges of isotopes are available.
• High sensitivity
• More effective
• Simple administration and isolation.
• Shows accurate results when enough metabolic time & technique is used.

• Position & Quantity of compound containing tracer isotope14 C marked glucose is used for glucose determination in the biological system.
• For different studies, different tracers can be used. For studies on nitrogen and amino acid, Labelled nitrogen give specific information than carbon.

3.2. Criteria for Tracer / Isotope Selection

Two types of isotopes are generally used for labeling:
Radioactive isotope: - [e.g. ^{1}H, ^{14}C, ^{24}Na, ^{42}K, ^{35}S, ^{35}P, ^{131}I] For biological investigation – C & H. For metabolic studies – S, P, and alkali and alkaline earth metals are used. For studies on protein, alkaloids, and amino acid - labelled N-atom.
Stable isotopes: - [e.g. ^{2}H, ^{13}C, ^{15}N, ^{18}O] Used for labeling compounds as possible intermediates in biosynthetic pathways. Usual method of detection are Mass spectroscopy [^{15}N, ^{18}O] and N.M.R spectroscopy [^{2}H, ^{13}C].

3.3. Steps Involved in Tracer Technique

1. Preparation of the labeled compound:
The labeled compound produces $^{14}CO_2$ by increasing it in the atmosphere. All carbon compounds are labeled 14 C. The labeled 3H (tritium) compound is commercially available. The labeling of tritium is effected by catalytic exchange in aqueous media by hydrogenation of an unsaturated compound with tritium gas. Tritium is pure β –low-intensity emitter and its radiation energy is lower than 14 C. Using organic synthesis:

$$CH_3MgBr + {}^{14}CO_2 \longrightarrow CH_3 + {}^{14}COOHMgBr + H_2O \longrightarrow CH_3 + {}^{14}COOH + Mg(OH)Br$$

2. Introduction of labelled compound into the biological system:
Root & stem feeding: This is the most common method of introduction of radio isotopes labelled reagents. Selection of the plant part depends upon the site of bio synthesis of the desired metabolite. e.g. tobacco alkaloids are mainly bio synthesized in the roots, hence root is the preferred site for feeding the labelled reagents.
Direct injection: This method of introduction of labelled reagent is employed for those plants which possess hollow stems of capsular fruits.
Wick feeding: In this method, cotton stands are passed through the plant stem. The terminal ends of these cotton strands are immersed into the reagent labelled with the radio isotopes.
Floating method: In this method, the chopped leaf pieces are made to float in the radio isotopes labelled reagent.
Spray method: This method is used for reagents which are readily absorbed from the leaf surface. e.g. steroids
Procedure: The plant is exposed to the organic compounds labelled with the radio isotopes for a short period of time using one of the above techniques. The bio syntheses occur sequentially and at each step radioactive products are formed. These products are isolated and identified. Then with the help of sequence of reactions, the entire pathway is traced out.
3. Separation and detection of compound: -
Geiger–Muller (GM) Counters: Geiger–Muller counter is a type of particle detector that measures ionizing radiation, e.g. alpha, beta particles or gamma rays, by ionization produced in low-pressure gas, usually helium, neon or argon with halogens added in the Geiger–Muller tube, which conducts electrical charges briefly when a particle or photon of radiation makes the gas conductive by ionization. This indictment has been detected in form of current pulse.
Liquid Scintillation Chamber: Principle: A scintillation detector or scintillation counter is produced when the scintillation detector is coupled to an electronic light sensor such as a photomultiplier tube (PMT) or a photodiode. A scintillator is a material that exhibits scintillation— a luminescence property that is stimulated by ionizing radiation. Samples shall be dissolved or suspended in a "cocktail" containing a solvent (aromatic organics such as benzene or toluene), typically some form of a surfactant, and small amounts of scintillators.
Method: The scintillation medium consists of a fluorescent solute and an organic solvent, such as benzene or toluene, in which the excitation takes place. The radiolabelled metabolite sample shall be dissolved in the scintillation medium. A specified quantity of solution obtained shall be placed in small, transparent glass vials. These vials are then placed in a liquid scintillation counter with 2 image multiplier tubes. Radiolabelled metabolites present a solution that emits beta particles. The energy of the beta particle is initially passed to the molecules of the solution and ultimately to the molecules of the solute. The solute is excited and disperses energy by emitting light. One beta particle corresponds to a single pulse of light. The pulse is measured using photomultiplier tubes.
Gas Ionization Chamber: The ionization chamber is the simplest of all gas-filled radiation detectors and is commonly used for ionizing radiation, including x-rays, gamma rays and beta particles. Conventionally, the term "ionization chamber" is used solely to describe those detectors that collect all the charges caused by direct ionization of the gas using an electrical field.
Mass Spectrophotometer: Mass spectrometry (MS) is an analytical technique used to measure the mass-to-charge ratio of charged particles. It is used to determine the mass of the particles, to determine the elemental composition of the sample or molecule, and to elucidate the chemical structures of the molecules, such as peptides and other chemical compounds.
NMR Spectrophotometer: NMR spectroscopy is a research technique that exploits the magnetic properties of certain atomic nuclei to determine the physical and chemical properties of the atoms or molecules they contain. It relies on the phenomenon of nuclear magnetic resonance and can provide detailed information on the structure, dynamics, reaction status and chemical environment of the molecules.
Autoradiography: Autoradiography is a tool for examining the distribution of radioactive material in a plant object, e.g. histological tissue, chromatography sheet. This method uses a photographic film or emulsion as an ionizing radiation detector. The specimen is in close contact with the emulsion for a period (exposure duration)
In this technique, a sample containing a radiolabelled metabolite is placed in direct contact with suitable photosensitive material such as x-ray (photographic) film for a specific period. The pattern of delivery of radioactive substances can be elucidated with the aid of the autograph collected.

4. Methods in Tracer Technique

4.1. Precursor product sequence:

In this technique, the presumed precursor of the constituent under investigation on a labelled form is fed into the plant and after a suitable time the constituent is isolated, purified and radioactivity is determined.

Disadvantage: - The radioactivity of isolated compound alone is not usually enough evidence that the particular compound fed is direct precursor, because substance may enter the general metabolic pathway and from there may become randomly distributed through a whole range of product.
Application: Stopping of hordenine production in barley seedling after 15 – 20 days of germination. Restricted synthesis of hyoscine, distinct from hyoscyamine in Datura stramonium. This method is applied to the biogenesis of morphine & ergot alkaloids

4.2. Double & multiple labelling:

This method gives the evidence for nature of biochemical incorporation of precursor arises double & triple labelling. In this method specifically labelled precursor and their subsequent degradation of recover product are more employed.

4.3. Competitive feeding:

If incorporation is obtained it is necessary to consider whether this in fact, the normal route of synthesis in plant not the subsidiary pathway.
Application: This method is used for elucidation of biogenesis of propane alkaloids. Biosynthesis of hemlock alkaloids (conline, conhydrine etc) e.g. biosynthesis of alkaloids of Conium maculactum (hemlock) using ^{14}C labelled compounds.

4.4. Isotope incorporation:

This method provides information about the position of bond cleavage & their formation during reaction. E.g. Glucose – 1- phosphatase cleavage as catalyzed by alkaline phosphatase this reaction occurs with cleavage of either C – O bond or P – O bond.

4.5. Sequential analysis:

The principle of this method of investigation is to grow plant in atmosphere of $^{14}CO_2$ & then analyze the plant at given time interval to obtain the sequence in which various correlated compound become labelled.
Application: $^{14}CO_2$ & sequential analysis has been very successfully used in elucidation of carbon in photosynthesis. Determination of sequential formation of opium hemlock and tobacco alkaloids. Exposure as less as 5 min. $^{14}CO_2$, is used in detecting biosynthetic sequence as – Piperitone, (-) Menthone, (-) Menthol in Mentha piperita

5. Application of Tracer Technique

- Study of squalene cyclization by use of 14C, 3H labelled mevalonic acid.
- Interrelationship among 4 – methyl sterols & 4, 4 dimethyl sterols, by use of 14C acetate.
- Terpenoid biosynthesis by chloroplast isolated in organic solvent, by use of 2-14C mevalonate.
- Study the formation of cinnamic acid in pathway of coumarin from labelled coumarin.
- Origin of carbon & nitrogen atoms of purine ring system by use of 14C or 15N labelled precursor.
- Study of formation of scopoletin by use of labelled phenylalanine.
- By use of 45Ca as tracer, - found that the uptake of calcium by plants from the soil. (CaO & CaCO2).
- By adding ammonium phosphate labelled with 32P of known specific activity the uptake of phosphorus is followed by measuring the radioactivity as label reaches first in lower part of plant, than the upper part i.e. branches, leaves etc.
- Tracing of bio-synthetic pathway of cyanogenetic glycoside “prunacine”; by incorporating 14 C into phenylaniline Interrelationship among 4 – methyl sterols & 4, 4 dimethyl sterols, by use of 14 C acetate.

6. Conclusion

The tracer technique mainly deals with the secondary metabolites and its applications in synthesis of in pharmacognosy. This technique involves the stable (1H2,6C13,7N15,8O18) and unstable (1H1,6C14) radioactive isotopes. This technique which utilizes a labelled compound to find out or to trace the different intermediates and various steps in biosynthetic pathways in plants, at a given rate & time and also deeply focused on the methods in tracer technique. Now a days this method is more useful to identify the secondary metabolites.

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Conflict of Interest

Authors declared that no conflict of interest

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