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Isotopic and Nuclear Analytical Techniques in Biological Systems: A Critical Study

PART X. ELEMENTAL ISOTOPE DILUTION ANALYSIS WITH RADIOACTIVE AND STABLE ISOTOPES

(Technical Report)

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Isotopic and nuclear analytical techniques in biological systems: A critical study—X. Elemental isotopic dilution analysis with radioactive and stable isotopes (Technical Report)

Synopsis – Isotope dilution is a method of chemical analysis based on the mixing (or dilution) of a radioisotope or a separated stable isotope with its natural isotope(s) in the sample. The activity ratio or isotopic ratio of the mixture defines the concentration of the analyte, which is a tremendous advantage for measurement since quantitative separation of the analyte is not required. The technique has a multitude of variations and has been combined with many classical and instrumental procedures used in analytical chemistry. The technique is noted for its accuracy. This review focuses on the application of the technique in the determination of elemental concentrations in biological systems.

Introduction

Isotope dilution analysis (IDA) was one of the first techniques to apply radioisotopes in chemical analysis, having been introduced by Hevesy and Hobbie in 1932 (ref. 1). The term "isotope dilution" was first applied in 1940 (ref. 2). Many variations of the basic technique have since been developed. The application of nonradioisotopes, that is, stable isotopes, can be considered one variation of IDA. Although the use of stable isotopes was initiated in the 1940's, first with C, N, O, and H (ref. 2-3), it was not until the 1950s that stable isotopes were more broadly applied with the increased availability of both mass spectrometers and electromagnetically separated isotopes. The theory of IDA and a discussion of its many variations have been published by Tölgyessy, Braun, and Kyrš (ref. 4). Table I lists more recent references, both reviews and applications of IDA.

Table 1. List of references reviewing IDA techniques and applications.

Techniques	References
Radiochemical IDA	
(a) Comprehensive review	5
(b) Environmental analysis (includes biologicals)	6
Isotope Dilution Mass Spectrometry	
(a) Comprehensive review	7,8
(b) General reviews	9,10
Application Reviews	
(a) Radiochemical IDA	11,12,13
(b) IDMS	14,15

The underlying principle behind the technique of IDA is the conservation of mass upon dilution. When radioisotopes are used, the conservation of activity is the manifestation of the conservation of mass. The dilution of the radioactive isotope by its non-radioactive counterpart results in the reduction of specific activity,

defined as the activity per unit volume or mass, in a conserved manner proportional to the original specific activity and the amount of analyte. When separated stable isotopes are used, the diluted natural isotopic ratio is related to concentration by the amount and isotopic composition of the separated isotope that was added. Mass (or atom) ratios are measured mass spectrometrically; the technique is known as isotope dilution mass spectrometry (IDMS). Other methods for measuring stable isotope ratios are possible, including optical, neutron activation analysis (NAA), and nuclear magnetic resonance (NMR). These methods are specific to certain isotopes and seldom applied.

The quantification of radioactive substances can be done by "inverse IDA" where known amounts of inactive material (carrier) are added to the sample. The same principles and formulas apply.

This review will concentrate on the determination of elemental concentrations in biological systems using IDA. The application of the technique to determination of organic and inorganic molecular species is specifically excluded, although the IDA technique is successfully used for these types of chemical analyses as well. For instance, the techniques of radioimmunoassay and radioreceptor analysis are probably the most common IDA related techniques (ref. 16).

The determination of inorganic elements in biological systems and a recognition of the variety of their biochemical functions is expanding greatly. There are at least three basic reasons for studying the interaction of inorganic elements with organisms. The first reason is to understand nutrition, that is, the assimilation and regulation of elements by the body. The second reason is to understand the role of elements in disease, especially in the diagnosis of disease states. The third reason is to monitor the impact of environmental toxins on organisms. This last activity is increasing because of more stringent occupational safety and health requirements and a heightened concern, in general, of the effects of low levels of toxic elements on the body.

Basic Principles Underlying Isotope Dilution Analysis

The basis of IDA is the mixing of accurately known amounts of stable or radioactive isotopes (called spikes or tracers) of an element with a sample. The principal requirement for accuracy is that the tracer and sample isotopes are fully equilibrated. The analysis will be in error if the sample is not completely dissolved. An error will also occur during separation of the analyte if the tracer and sample are not chemically equivalent and differing separation efficiencies exist for the differing chemical forms. The principal advantage of IDA is that separations need not be quantitative; an activity ratio or isotope ratio defines the concentration.

Classical IDA (or direct IDA, DIDA) using radioisotopes requires isolation of an amount of a chemically pure compound measurable by a second technique, which limits its sensitivity to the sensitivity of this second technique. Standard separation procedures (e.g. precipitation, distillation, solvent extraction) have all been applied and mass (or yield) determinations are accomplished using standard analytical methods (e.g. gravimetry, colorimetry, atomic spectroscopy). The general equation for classical IDA is:

$$W_x = W_0 \left(\frac{S_0}{S_2} - 1 \right) = W_0 \left(\frac{A_0 W_2}{W_0 A_2} - 1 \right) \qquad (1)$$

where, W_x = weight of unknown;
W_0 = weight of spike added;
A_0 = activity of spike added;
and, S_0 = specific activity of spike added = A_0/W_0.
After dilution, W_2 = weight of analyte isolated;
A_2 = activity of analyte isolated;
and, S_2 = specific activity of analyte isolated = A_2/W_2.

The general equation results from the relationship for the conservation of activity:

$$\frac{A_2}{W_2} = \frac{A_0}{W_x + W_0} \quad (2)$$

If W_0 is small relative to W_x, then equation (1) is simply:

$$W_x = \frac{A_0 W_2}{A_2} \quad (3)$$

Substoichiometric IDA avoids the requirement for determination of the amount isolated for the measurement of specific activity after dilution. A ratio of activities is measured when the exact same amount of material is isolated from a sample and a standard. A suitable reactive reagent is added substoichiometrically (reagent:analyte << 1) to the sample and standard. The reaction products then are isolated. The absolute amount of reagent need not be known, nor the extent of the reaction, as long as the standard and sample react and are isolated with equal efficiency. An additional advantage of this technique is provided by the selectivity of the reagent toward reaction with the analyte. It is obvious that this method is dependent upon the availability of a suitable reactive reagent.

Sub-superequivalence methods of IDA avoid the requirement in substoichiometric IDA that the efficiency of separation be the same for sample and standard, which may be difficult if the standard and sample have very different compositions. In the basic method of this class, a series of solutions is prepared in which the individual solutions contain the same amount of sample and radioactive spike but are isotopically diluted with increasing amounts of stable carrier. A second set of solutions is prepared which contains multiple amounts of the sample and radioactive spike mix in the first series, but no stable material. With all solutions brought to the same volume, a substoichiometric amount of reagent is added to each solution; the products are isolated; and, the activity ratio versus carrier added is plotted to quantify the analyte. This technique is IDA combined with the standard addition method.

Isotope dilution mass spectrometry is simply applied. The enriched stable isotope(s) are added to the sample and equilibrated. Measurement of the isotope ratio mass spectrometrically is used to determine the concentration. Separation of the analyte from the sample is not required but is often done for two reasons: to achieve efficient ionization and to eliminate isobaric interferences that can occur. IDMS can be accomplished using the many variations of mass spectrometers available and the many possible ionization sources, including thermal ionization, secondary ionization, plasma source ionization, and electron impact ionization. The general equation for IDMS is:

$$C_x = \left(\frac{C_s Wt_s}{Wt_x}\right)\left(\frac{I_{1s} - R_m I_{2s}}{R_m I_{2x} - I_{1x}}\right) \quad (4)$$

where, C_x = concentration of unknown;
C_s = concentration of spike;
Wt_s = weight of spike mixed with sample;
Wt_x = weight of sample;
I_{1s} = abundance of isotope I_1 in spike;
I_{2s} = abundance of isotope I_2 in spike;
I_{1x} = abundance of isotope I_1 in sample;
I_{2x} = abundance of isotope I_2 in sample;
R_m = atom ratio of I_1/I_2 after dilution.

The general equation results from the mass balance equation:

$$R_m = \frac{C_x Wt_x I_{1x} + C_s Wt_s I_{1s}}{C_x Wt_x I_{2x} + C_s Wt_s I_{2s}} \quad (5)$$

where $C_x Wt_x I_{1x}$ is the number of moles (or atoms) of isotope I_1 in the sample, $C_s Wt_s I_{2s}$ is the number of moles (or atoms) of isotope I_2 added from the spike, etc.

Merits and Limitations to IDA

The main advantage of IDA is the luxury of using nonquantitative isolation procedures in the separation of analyte. This advantage provides considerable flexibility in the design of separations, which can be optimized for simplicity, robustness, speed, cost, or some other analytical parameter. IDMS is a highly accurate technique because there are minimal sources of potential systematic error.

The principle limitation to IDA is the availability of a suitable spike or tracer. The half-life and type of radiation emitted by a radiotracer is very important, as is the purity. The half-life must be long enough so that sufficient activity is available during the analysis for good counting statistics. However, too long half-lives can be a problem because of low specific activities, and storage and disposal problems. The type of radiation is important primarily in relation to the ease of measurement. There are suitable ($t_{½}$ > 10 min) radiotracers available for most elements in the periodic table. The exceptions are He, Li, B, N, O, and Ne. Separated stable isotopes are available for some 80% of the elements of the periodic table. In addition, some of the 19 mononuclidic elements have long-lived radioisotopes, e.g. ^{129}I and ^{230}Th, which are suitable for IDMS.

Practically, IDA is done in established laboratories that have the resources available and wish to apply these resources in an optimal way. The increasing costs of maintaining a laboratory that is equipped, licensed and regulated for radiochemical use and the cost of mass spectrometry instrumentation required for IDMS are certainly barriers for expansion of the IDA technique in the analytical field. The costs of tracers have been cited as a limitation to IDA. This limitation is less true for stable isotopes which can be calibrated and stored for long periods of time with no loss of usefulness. Paradoxically, as the analytical measurement becomes more difficult, that is, when the level to be determined becomes increasingly smaller, the amount of stable spike required becomes smaller as well and negligible relative to other components of the analysis cost.

Sensitivity in IDA varies according to both the technique and the tracer isotope. In direct IDA, the limit is the smallest amount that can be determined or purified. The specific activity and isotopic purity of the tracer is the major limit to sensitivity in substoichiometric IDA, although this technique also is limited by finite equilibrium constants and incomplete and variable reaction efficiencies at very low concentrations of analyte. Theoretical detection limits for 38 elements with readily available, beta-emitting isotopes of reasonably long half life have been tabulated and range from 10^{-8} to 10^{-16} g (median 10^{-11} g) (ref. 17).

The sensitivity for ultratrace measurement when chemical separations are done and significant amounts of reagents are used is often limited by the blank, or contamination that occurs from the presence of the analyte in the reagents. This fact is especially true for IDMS, where typically very high sensitivities can be achieved instrumentally for most elements. Thus, procedures which minimize sample handling and amounts of reagents used and which use ultrapure reagents in laboratory clean rooms are required to reach the lowest limits of detection. The fact that separations are not specifically required in IDMS can help to minimize blank and be an advantage. Blank levels can vary considerably from pg (10^{-12} g) to μg (10^{-6} g).

One of the limitations of IDA is its general application as a single element technique. However, the difficulties with preparing, storing, and using multielement radiotracers are not applicable to the preparation and use of multielement stable isotopic tracers, which are routinely used. Multielement instrumental methods are not as widely applied, but the advent of new mass spectrometric instrumentation promises more widespread application of multielement IDMS methods.

Exemplary Applications in Biological Systems

Applications of IDA in inorganic analysis have recently been tabulated (ref. 5), although it has been observed that application of radioisotopic IDA in quantitative analysis has "completely lost ground to competitive methods" (ref. 18). However, two examples which combine IDA procedures with one of these competitive methods, neutron activation analysis (NAA), illustrate the continued power and utility of the technique as well as two roles played by inorganic analysis of biological systems. This work of Turel and colleagues used radiochemical NAA, inverse IDA, and substoichiometric separations to quantify the analytes. One application was a comparison of the levels of 13 elements in normal, benign, and cancerous brain tissue (ref. 19). The second application was the determination of Hg in various biological samples (ref. 20). The first application illustrates the search for trace element indicators of disease; the second application illustrates the interest in quantification of environmental toxins in biological systems.

IDA serves a unique role in quantitative analysis of biological systems which results from the precision and accuracy of IDMS, and that role is as a "definitive method" in clinical chemical analysis. A hierarchy of measurement methods has been established in this field to benchmark accuracy. The hierarchy goes from the routine methods applied in the clinical laboratory to a carefully studied reference method to the definitive method. IDMS is the definitive method for the serum electrolytes potassium, chloride, lithium, magnesium, and calcium (ref. 21-22).

Another example illustrates how the accuracy and sensitivity of IDMS can be applied to understanding the interaction of trace and toxic elements with biological systems. It has been observed that the "normal" concentration of uranium in urine has declined by several orders of magnitude over the years (ref. 23). This decline is not a real change, in all probability, but a reflection of the improvement in analytical methods. The IDMS results of Kelly and coworkers of the U in the urine of young children represent the lowest levels reported (ref. 24). The establishment of an accurate baseline for non-occupationally exposed people is necessary to put regulatory requirements for occupational exposure in the proper perspective.

IDA is applied to problems broader than chemical analysis. One example is its use in the determination of blood volume. The radioisotope ^{51}Cr has been used in clinical practice since 1950 for this application (ref. 25). The technique relies on the extraction of blood, labelling with ^{51}Cr, and reinjection into the body. Since the labelled blood is chemically stable, a second sampling after dilution/equilibration will provide the blood volume information. Because of the increased restrictions on using radioisotopes with humans, and inappropriateness of using them with pregnant women and infants, applications of stable isotopes for this measurement have been developed. Zeisler and Young have published a method based on NAA, ^{50}Cr stable isotope dilution, and measurement of ^{51}Cr induced activity (ref. 26). Similarly, mass spectrometric measurements of the stable isotopes directly are also possible and our being pursued (ref. 27).

The application of radiotracers and stable isotopes has a long established tradition in the study of metabolism and nutrition, where the fate of ingested isotopes are traced versus time (ref. 28). This application is not IDA based, since the primary goal is the understanding of the kinetics of the complex process of tracer equilibration with the natural stable isotopes in the body. IDA can be used, however, as the analytical technique to quantify the absolute abundances of the isotopes excreted in the experiment. An example is a method published by Ting and Janghorbani using stable isotopes of iron: ^{58}Fe is the "in vivo spike"; ^{57}Fe is the "in vitro spike". Isotope ratio measurements are made by inductively coupled plasma mass spectrometry (ICP-MS).

Future Trends of IDA Applied to Biological Systems:

IDA must be considered a mature method, especially in regards to inorganic elemental measurements using radiotracers. Many of the recent applications are in the field of organic measurement where quantification is difficult and independent methods of measurement are limited. The inverse IDA technique is routinely used for measurement of radioisotopes because of the sensitivity. IDMS has not been widely applied to biological systems because isotope ratio mass spectrometers are concentrated in the geological, nuclear, and standards communities. The one exception to this generalization is the stable isotopes in nutrition field cited above.

Often organic mass spectrometric instrumentation and techniques (e.g. GC-MS) have been adapted for isotope ratio measurement in this field.

One positive trend for IDA is the expansion of the use of inorganic mass spectrometers in the field of analytical chemistry. This expansion can be tied directly to the invention of the ICP-MS, which was only introduced in 1983. The latest estimate is that over 600 of these instruments have been installed worldwide. The technique is growing rapidly because of the versatility and sensitivity of the ICP-MS relative to other inorganic analytical techniques. And, the leading practitioners are teaching that IDMS is the quantification method of highest accuracy for ICP-MS. Thus, although IDMS may not be practiced widely now, the potential exists for its greater and routine application in the future.

The increasing need for reliable and sensitive measurement of inorganic species, especially toxic elements, in biological systems assures the continued application of IDA to these problems. Because of its accuracy, IDA is well suited to benchmarking other analytical techniques, either through certification of standards or definitive measurements. Quality in performance is a basic concern in the scientific laboratory. IDA measurements can directly address this concern.

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