[14] Accelerator Mass Spectrometry for Biomedical Research

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Abstract

Accelerator mass spectrometry (AMS) is the most sensitive method for detecting and quantifying rare long-lived isotopes with high precision. In this chapter, we review the principles underlying AMS-based biomedical studies, focusing on important practical considerations and experimental procedures needed for the detection and quantitation of ^{14}C- and ^{3}H-labeled compounds in various experiment types.

Introduction

AMS is the most sensitive method available for detecting and quantifying rare long-lived isotopes with high precision. This technique is widely employed in the earth and environmental sciences for purposes such as radiocarbon dating and studying the circulation of the world's oceans (Vogel *et al.*, 1995). It was not until the late 1980s that AMS was first used in biological research. Since then, it has been used primarily to investigate the absorption, distribution, metabolism, and excretion of radiolabeled drugs, chemicals, and nutrients, as well as in the detection of chemically modified DNA and proteins in animal models and humans. Newer applications of AMS include an isotope-labeled immunoassay (Shan *et al.*, 2000) and attomole-level sequencing of ^{14}C-labeled protein, achieved by coupling Edman degradation with AMS detection (Miyashita *et al.*, 2001). The high sensitivity of AMS measurements translates to the use of low chemical and radioisotope doses and relatively small sample sizes, which enables studies to be performed safely in humans, using exposures that are environmentally or therapeutically relevant while generating little radioactive waste.

Most biomedical AMS studies completed have employed carbon-14 as the radiolabel, although the capability exists for detecting other isotopes including ^{3}H, ^{26}Al, ^{41}Ca, ^{10}Be, ^{36}Cl, ^{59}Ni, ^{63}Ni, and ^{129}I. Both ^{14}C and ^{3}H are commonly used in tracing studies because they can be readily incorporated into organic molecules, either synthetically or biosynthetically. In this chapter, we review the principles underlying AMS-based biomedical studies, focusing on important practical considerations and experimental

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procedures needed for the detection and quantitation of ^{14}C- and ^{3}H-labeled compounds in various experiment types.

Methodology

AMS is 10^3–10^9 fold more sensitive than the decay counting methods routinely employed in biological studies involving radioisotopes (Turteltaub and Vogel, 2000). A detailed explanation of the differences between decay counting and AMS is presented in Chapter 13 by Vogel *et al.* Decay counting indirectly predicts the number of isotope nuclei present by measuring decay events. For ^{14}C and ^{3}H isotopes, this is an inefficient process, dependent on the length of time the sample is counted and the number of nuclei present. AMS directly quantifies each individual isotopic nucleus and is independent of these variables. This results in improved sensitivity, which means AMS biomedical studies can be performed with isotope doses 100–1000 times lower than those traditionally used and sample sizes can be 10^5–10^6 fold lower.

Instrumentation

A variety of instrument designs exist depending on the accelerating voltage and measurement requirements such as precision, resolution and the isotope range. At Lawrence Livermore National Laboratory (LLNL), a multipurpose 10 MV system (Fig. 2A) is used for the analysis of a wide range of isotopes from biomedical and earth and environmental science studies. This AMS machine includes two mass spectrometers separated by an electrostatic accelerator, through which negative ions are accelerated to high energies (Fig. 1). Individual graphite (for ^{14}C) or titanium hydride (for ^{3}H) pellets in aluminum holders, derived from the sample material, are bombarded with a large current of positive cesium ions (3–10 keV), which produces negatively charged elemental and molecular ions (Vogel, 1992). These undergo an initial separation through a low-energy mass spectrometer. Ions are then accelerated to the positive high-voltage terminal (3–10 MV) at the midpoint of the tandem accelerator where they pass through a thin carbon foil or gas, which strips off electrons, forming positive ions and causing dissociation of interfering molecular isobars. The charge state is dependent on ion velocity. With a 7 MV terminal, most C^- ions take on a 4+ charge and are then further accelerated back to ground potential in the second half of the accelerator. After emerging from the accelerator, the rare ions are separated from the abundant ions by a high-energy mass spectrometer and crossed electric and magnetic fields (Wein filter).

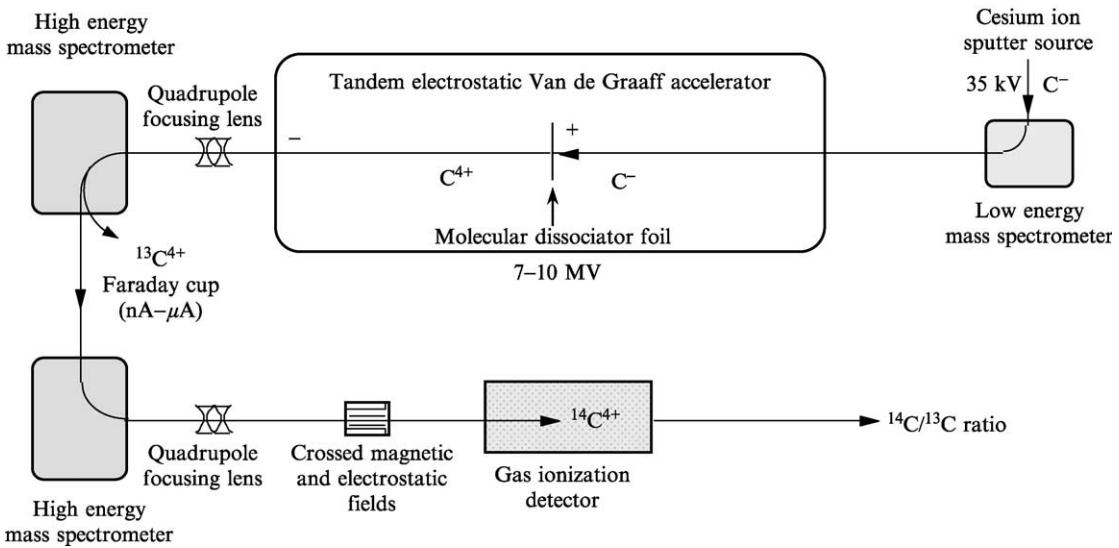

FIG. 1. Diagram of the 10 MV accelerator mass spectrometry (AMS) system at the Lawrence Livermore National Laboratory.

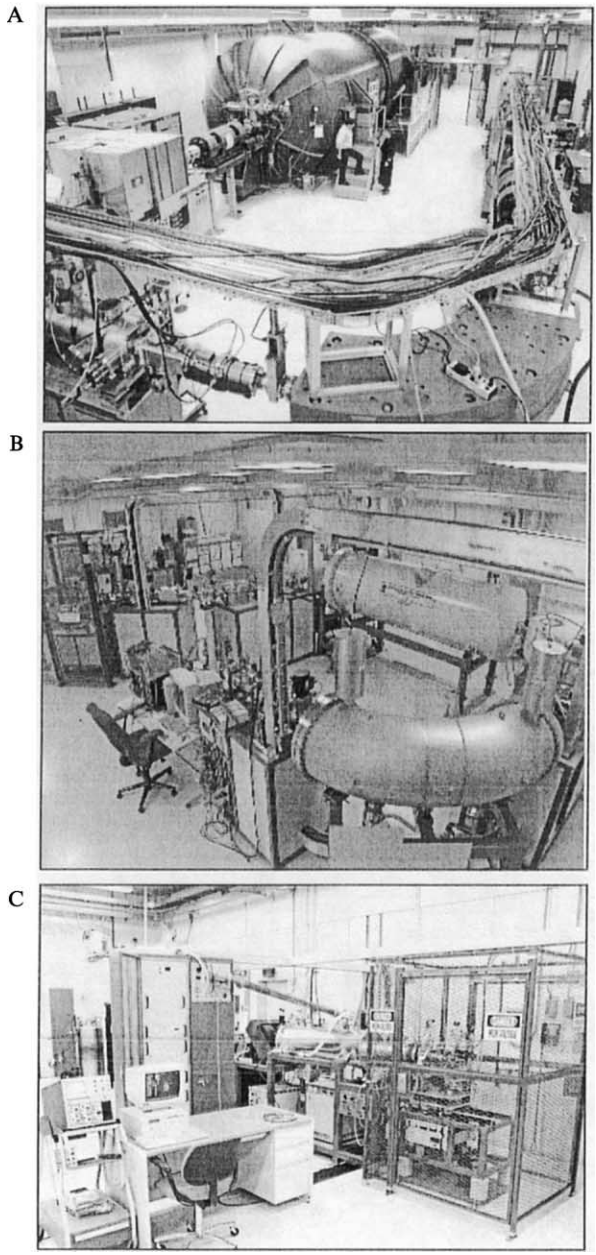

FIG. 2. Accelerator mass spectrometers in use at the Lawrence Livermore National Laboratory. (A) The high-energy 10 MV instrument used by multiple researchers for the measurement of various isotopes. (B) The 1 MV accelerator mass spectrometry (AMS) system dedicated to the analysis of ^{14}C in biomedical samples. (C) The compact tritium AMS system.

Isotopic ions are then individually identified through characteristic energy loss and counted in a gas ionization detector.

AMS does not determine absolute concentrations of radioisotopes; each measurement is an isotope ratio: the ratio of a rare isotope to a stable isotope of the same element. Expressing results as a ratio corrects for fluctuations in ion transport through the AMS system. For ^{14}C or ^{3}H analysis, ^{13}C or ^{1}H is measured sequentially with the radioisotope of interest (^{14}C or ^{3}H, respectively) as a current in a Faraday cup. The isotope ratios are then compared with a set of standards with known ^{14}C or ^{3}H content to determine the precise amount of ^{14}C or ^{3}H in the sample analyzed.

At the LLNL facility, a much smaller 1 MV spectrometer was built, designed specifically for measuring ^{14}C in samples from biomedical studies (Fig. 2B). This lower voltage spectrometer, which can measure more than 300 samples a day with precisions of 3%, is now used for quantifying ^{14}C in all biological samples at LLNL (Ognibene *et al.*, 2002). A compact AMS system dedicated to the measurement of ^{3}H has also been developed (Roberts *et al.*, 2000) (Fig. 2C). The system uses a radiofrequency quadrupole (RFQ) LINAC, which has a compact size (<1.5 m) and is able to accelerate all three hydrogen isotopic species to energies sufficient for measurement using a simple magnetic spectrometer. Other compact lower voltage AMS instruments are also being developed with variations in ion source and sample introduction (Hughey *et al.*, 1997).

Sample Preparation: Methodology and Practical Considerations

The most important considerations in preparing samples for AMS analysis are the predicted amount of radioisotope in each sample, preventing contamination and knowing the sources and amounts of any carbon introduced during processing. Numerous precautions need to be in place throughout the procedure to ensure the amount of isotope present is within the dynamic range of the spectrometer and to minimize the potential for contamination, to ensure that the isotope detected in the sample is associated with the labeled compound under investigation.

The sample of interest, for example, blood, DNA, or high-performance liquid chromatography (HPLC) fractions, must be converted to a form that is compatible with the ion source of the instrument. Most ^{14}C samples are measured as graphite and ^{3}H samples as titanium hydride. The advantages of analyzing samples in a solid state rather than as a gas include a lower risk of cross-contamination in the ion source, shorter memory effects when a ''hot'' sample is encountered, and higher sample throughput due to more efficient ionization. Solid samples can be prepared at any location and sent

to an AMS facility for analysis. This allows multiple researchers working on many experiments in various locations to use the instrument simultaneously. Solid samples can be left in the ion source for as long as necessary to accumulate sufficient events in the detector to attain the required measurement precision and can be saved for reanalysis if desired. However, the ability to directly couple an AMS instrument to an analytical HPLC, CE, or gas chromatograph (GC) system would provide a powerful and necessary technique for biomedical research, because it would enable online analysis of components in a sample without the need for prior separation and sample preparation. A prototype system has been described, which interfaces a GC to an AMS instrument (Hughey *et al.*, 2000). Following GC separation, organic compounds eluting from the column are converted to CO_2 in a combustion chamber before entering the ion source. In addition, nonvolatile samples, including eluent from an HPLC system, can also be analyzed using a laser-induced combustion interface in which liquid is deposited into a bed of CuO powder (Liberman *et al.*, 2004). Heating the matrix locally with a laser causes sample combustion and the resulting CO_2 is transported in a stream of carrier gas into the ion source of the AMS instrument. Ultimately, the authors intend this system to be used for the online separation and detection of both ^{14}C- and ^{3}H-labeled compounds using various chromatographic techniques.

The standard procedure for the production of graphite or titanium hydride involves oxidation of the crude sample to a gaseous mixture including carbon dioxide and water (Fig. 3). Carbon dioxide is then cryogenically extracted and reduced to form filamentous graphite, and water is reduced to hydrogen gas and adsorbed on to titanium, forming titanium hydride. The isolated sample, typically in the form of a DNA or protein solution, HPLC fraction, or wet tissue, is transferred to a quartz tube, which is baked before use to remove all bound carbon. This tube, which must be handled with disposable forceps to prevent contamination of the exterior, is placed in an outer borosilicate glass tube to facilitate handling, and the volatile components of the sample are removed by vacuum centrifugation. Copper oxide in wire form is added, and the inner quartz tube transferred to a quartz combustion tube, which is evacuated and sealed using an oxyacetylene torch. The sample is then oxidized by heating in a furnace at 900° for 2 h, which produces CO_2, N_2, and H_2O (Vogel, 1992). For production of graphite, the combustion tube is attached to a Y-shaped disposable plastic manifold, the other ends of which are connected to a reaction tube, containing reagents for the reduction of CO_2 to elemental carbon (titanium hydride, zinc, and cobalt powders) and a vacuum line, as shown in Fig. 4. The next step is to cryogenically separate CO_2 from the

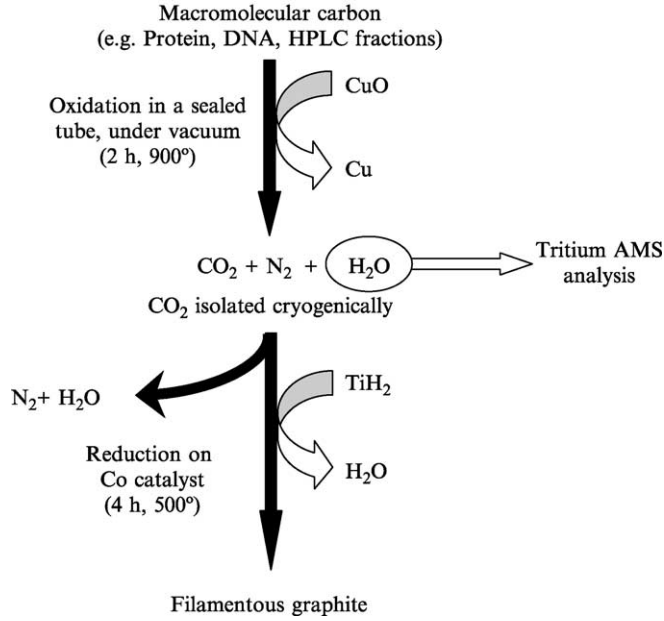

FIG. 3. Stages of sample combustion and graphitization.

other oxidation products and trap it in the reaction tube. This involves first evacuating the reaction tube, then clamping off the vacuum. The combustion tube is placed in a dry ice–isopropanol slurry, which condenses out the H_2O. The end of the combustion tube is broken open, allowing transfer of CO_2 over to the reaction tube. This tube is placed in liquid N_2, which freezes out and traps the CO_2. Finally the hose clamp is removed to evacuate N_2 gas, and the reaction tube is sealed. The samples are baked in a furnace for 4 h at 500°. The CO_2 is reduced to graphite by zinc and titanium hydride and deposits on the cobalt catalyst contained in a smaller inner tube of the reaction tube.

The sample-preparation stage is the rate-limiting step in AMS studies, but a method has been developed for preparing graphite from CO_2 gas in septa-sealed vials (Ognibene *et al.*, 2003). This approach increases sample throughput and is less complex than the standard protocol, because it does not require the use of a plastic manifold or torch sealing of the transferred combustion products. Instead, the combusted sample is transferred to a septa-sealed vial containing zinc dust and an inner quartz tube with iron

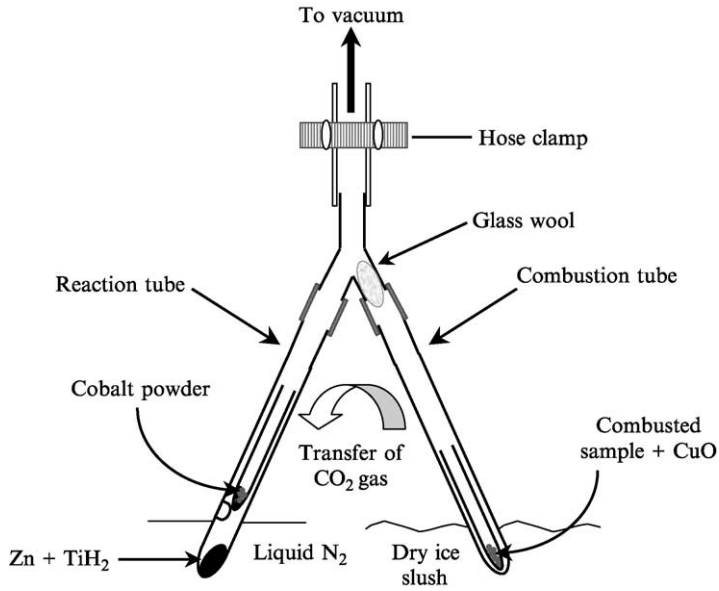

FIG. 4. Transfer of CO_2 from the combustion tube to the reaction tube in the preparation of graphite for ^{14}C analysis.

powder, via a needle connected to a Luer-Lok stopcock. The vial is kept in liquid nitrogen to cryogenically trap the CO_2 and H_2O while noncondensing gases are removed by vacuum pump. The needle is removed from the vial, which is then placed in a block heater and held at 500°, reducing the CO_2 to graphite. This newer protocol results in lower process backgrounds and allows for preparation of smaller sized samples. It has been adopted as the routine method at the LLNL for preparing biological samples for ^{14}C analysis by AMS and is expected to form the basis for an integrated automated system, aimed at considerably increasing sample throughput.

For analysis of ^{3}H, water from the combustion process is trapped using a dry-ice isopropanol bath and then reduced to hydrogen gas using zinc. The resulting hydrogen gas is adsorbed onto titanium to produce titanium hydride (Chiarappa-Zucca *et al.*, 2002; Roberts *et al.*, 1994). For AMS measurement, the prepared graphite or titanium hydride is packed into individual sample holders and placed in a sample wheel. Samples are measured at least three times, up to a maximum of seven, until the measurement precision is less than 3%.

The high sensitivity of AMS measurements means that a number of precautions must be taken during sample preparation to prevent

contamination. Laboratory surfaces and equipment are routinely swiped using a glass filter wetted with ethanol, to monitor for removable contamination. Airborne ^{14}C contamination is detected using sorbent carbon, such as graphitized coal or fullerene soot mixed with fine metal powder and packed into AMS sample holders. These samples are left in the laboratory for several days or weeks and then measured by AMS to check for contamination during this period. These methods report airborne and surface contamination after the event, alerting users to a problem, and supplement the numerous procedures that are in place to prevent contamination of samples and the laboratory environment; each stage of the sample preparation process is physically segregated, according to the activity level being handled. To avoid sample contamination during handling, disposable plastic labware is used whenever possible, bench covering is changed frequently, and only specifically designated equipment (centrifuges, freezers, HPLC instruments, etc.) is used for AMS work (Buchholz *et al.*, 2000).

Data Manipulation

Living entities maintain a natural level of ^{14}C because of the relatively constant production of this isotope in the upper atmosphere. This level, referred to as ''Modern,'' corresponds to 97.6 amol of ^{14}C/mg of total carbon, or 6.11 fCi ^{14}C/mg of carbon. AMS is used in biomedical studies to measure the increase in ^{14}C or ^{3}H above natural background in a particular sample, which is due to the presence of a ^{14}C- or ^{3}H-labeled parent compound or related derivative.

Samples that generate approximately 0.5–1.0 mg of total carbon or 2.0 mg of water upon combustion are optimal for AMS analysis using the methods described earlier. Samples containing sufficient natural carbon or ^{1}H are measured neat, whereas smaller samples are supplemented with a precise amount of carrier that provides sufficient carbon or water for optimal sample preparation. An ideal carrier has radioisotope concentrations well below the levels found naturally in living organisms (<1% contemporary isotope abundance). Tributyrin (25 μl of a 40 mg/ml solution in methanol or 1 μl neat tributyrin providing 0.6 mg of carbon) is often used as the carrier because it is a nonvolatile liquid and contains depleted levels of ^{14}C and ^{3}H. We have found measurements of tributyrin samples to be highly reproducible. With every series of carrier-supplemented samples submitted for AMS analysis, a set of three or four samples containing just the carrier is also measured.

To determine the exact amount of ^{14}C or ^{3}H in a sample by AMS measurement, the complete carbon or hydrogen inventory must be known.

For biological samples, this information can be acquired by elemental analysis or using published reference values for a particular tissue type. Calculation of ^{14}C or ^{3}H content is easiest for samples that can be obtained in quantities yielding in excess of 1 mg of carbon or 2 mg of water, such as blood, whole tissue, and isolated proteins. In this case, the excess level of ^{14}C or ^{3}H in a sample, above that of an undosed control, is due to the presence of ^{14}C- or ^{3}H-labeled compound. If carrier is added to the samples, this must also be accounted for in the calculations by subtracting the amount of radioisotope contributed by the carrier and that contributed by the natural radioisotopic abundance of the actual sample. To do this, the precise amount of carrier added and the exact mass of biological material in the sample being analyzed must be known. Values are then converted to an appropriate form, such as picograms of radiolabeled compound per gram of tissue or tissue isolate (e.g., DNA or protein) using the specific activity, molecular weight of the labeled compound, and the %w/w carbon present in the tissue. For a detailed account of data handling, including example calculations, readers are referred to Chapter 13 by Vogel *et al.*, in this volume.

Applications

AMS can be used to trace the fate of any molecule in *in vitro* systems or whole organisms if it is labeled with an isotope appropriate for AMS analysis. Studies have been undertaken with a variety of drugs, environmental carcinogens, and micronutrients, to determine the kinetics of absorption, distribution, and excretion, to identify and quantify metabolites, and to assess DNA or protein-binding capability.

The sensitivity of AMS measurement gives this technique a number of major advantages over other methods for the detection of isotopes. Importantly, because only low doses of chemical and radioactivity are required, studies can be performed with levels of chemicals equivalent to therapeutic or environmental exposures. This is a significant feature because the biological effects observed at high doses may not extrapolate to the low doses humans typically encounter. Furthermore, with safer, low radioisotope doses, it is possible to perform studies in humans. Doses used in human protocols range from 10 nCi/person for metabolism and mass balance studies up to 50 μCi/person for DNA binding studies. These are within the range that corresponds to natural background levels of radiation that individuals are exposed to during everyday life.

Although this chapter concentrates on ^{14}C- and ^{3}H-AMS, many other radioisotopes are increasingly being used in biological studies. Bone

calcium metabolism has been studied in humans administered ^{41}C orally, as the carbonate (Freeman *et al.*, 2000), and this isotope has been investigated as a tracer for calcium uptake and deposition in cardiac ischemia (Southon *et al.*, 1994). A better understanding of aluminum absorption, distribution, and clearance in healthy patients and a group with Alzheimer's disease has been gained through AMS studies using ^{26}Al (Kislinger *et al.*, 1997; Moore *et al.*, 2000; Talbot *et al.*, 1995). In addition, uptake of silicic acid has been determined by ^{32}Si-AMS (Popplewell *et al.*, 1998). Other isotopes are also being developed for use in biomedical studies, including ^{10}Be and isotopes of plutonium.

AMS Determination of Radioisotope Concentrations: Pharmacokinetic Studies

One of the simplest and most common applications of biological AMS has been in the determination of absorption, distribution, metabolism, and excretion characteristics of isotope-labeled compounds in animal models and humans. Some unique aspects of AMS pharmacokinetic studies include the ability to determine long-term kinetics and metabolism using low doses, several months after isotope administration. Detailed pharmacokinetic data require frequent sampling, which is made possible with AMS detection, by virtue of the small sample sizes needed for analysis. AMS has been used to establish the kinetics of β-carotene uptake and plasma clearance in a human volunteer who received a single dose of ^{14}C-β-carotene obtained from ^{14}C-labeled spinach (Dueker *et al.*, 2000). Plasma concentrations of β-carotene and its metabolites were determined at intervals over a 7-mo period and required just 30 μl of plasma/analysis. Such complete investigations would not be possible using other methods that lack the necessary sensitivity to detect compounds and metabolites months after dosing.

To quantify concentrations of ^{14}C- or ^{3}H-labeled compounds and their derivatives, wet tissue samples (5–10 mg) or aliquots of fluid, such as blood, plasma, or urine (typically 10–200 μl), are prepared for analysis by placing them in quartz sample tubes and drying in a vacuum centrifuge to remove water before combustion. These are then processed as usual and measured by AMS. In these types of experiments, radioisotope concentrations can be relatively high, particularly with samples taken at early time points, so it is very important to screen samples before AMS analysis to ensure the samples do not contain too much radioisotope. This is done by liquid scintillation counting (LSC) an aliquot of each sample, using an amount equivalent to that intended for AMS analysis. The AMS measurement

range is 0.01–100 Modern, which equates to approximately 0.1–1350 dpm/g of carbon. Consequently, if radioisotope can be detected above background levels using LSC, the sample must be diluted before AMS analysis. If the signal is below or at the limit of detection, analysis can be performed.

A new application of AMS, made possible by advances in tritium detection (Chiarappa-Zucca *et al.*, 2002; Love *et al.*, 2002), is dual isotope-labeling studies. Dingley *et al.* (1998, 2003) have demonstrated that two independent compounds can be traced if one is labeled with ^{14}C and the other ^{3}H. For example, the liver concentrations of two heterocyclic amines ^{3}H-PhIP and ^{14}C-MeIQx were determined when the compounds were administered to rats individually and in combination, to ascertain whether co-administration affected bioavailability (Fig. 5). The methodology permits liver samples from a single source to be assayed in parallel for the presence of either ^{14}C or ^{3}H. Furthermore, simultaneous extraction of CO_2 and H_2O from the same sample is also now possible so that both isotopes can be quantified using just one sample, thereby increasing sample throughput and decreasing the amount of material required (Chiarappa-Zucca *et al.*, 2002). This will enable animal and human studies to be carried out involving mixtures of compounds, to more closely mimic typical human exposures.

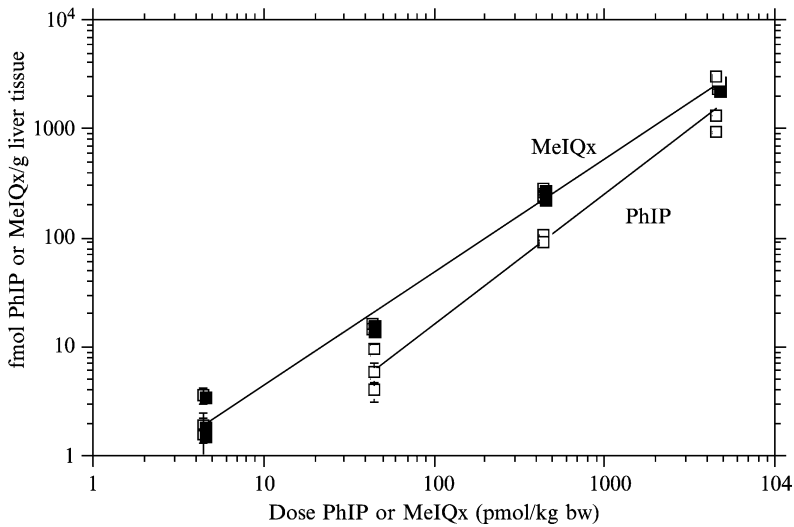

FIG. 5. Concentration of ^{3}H-PhIP and ^{14}C-MeIQx in the liver tissue of rats administered a dose range of either ^{3}H-PhIP or ^{14}C-MeIQx alone or in combination.

HPLC-AMS

AMS measurements provide no structural information, so any sample characterization or identification must be performed before sample preparation, using chromatographic (TLC, HPLC, size exclusion, and affinity) or electrophoresis (gel and capillary) separation techniques. Coupling an HPLC step to AMS detection affords both specificity and high sensitivity, in an approach that has been used for metabolite profiling and quantification (Buchholz *et al.*, 1999; Dueker *et al.*, 2000; Malfatti *et al.*, 1999), as well as the identification of chemically modified nucleosides (Mauthe *et al.*, 1999) in humans and animal models where other techniques are not sensitive enough to detect the low levels formed. Metabolites can be extracted and analyzed from a variety of biological matrices such as plasma, urine, tissues, and milk and a full metabolite profile can be obtained using just 100 μl of urine (Turteltaub and Vogel, 2000). Before beginning an experiment, aliquots of HPLC buffers, solvents, and any standards that might be run should be measured by AMS to check for contamination. Importantly, the HPLC solvents must be compatible with the graphitization process, in particular buffers containing sodium salts should not be used. Ideally, the buffer should not introduce an extra source of carbon, although it is sometimes possible to use such buffers if an isocratic system is employed and the amount of carbon in each fraction is constant throughout the run. In this situation, it is important that the samples do not contain too much carbon, because this can cause the tubes to explode during the combustion step. Samples are separated by HPLC and individual fractions collected at known intervals, typically every 20–60 s. In most cases, the fractions usually contain very little carbon after removal of the solvent, and tributyrin is added to each fraction to provide a fixed amount of carbon, sufficient for AMS measurement. The quartz sample tubes have a maximum capacity of approximately 300 μl, limiting the volume that can be submitted. Therefore, in situations in which the radioisotope content is expected to be low, fractions should be concentrated before this stage. Alternatively, 300-μl aliquots can be dried down repeatedly in the quartz tubes, but this can be time consuming. Performing and analyzing a control run of unlabeled material processed in an identical manner to the test sample is vital because the background ^{14}C or ^{3}H level of the system determines the detection limit of the method. The concentration of ^{14}C or ^{3}H in each fraction is determined from the isotope ratio and is used to reconstruct a chromatogram. Individual metabolites and compounds can then be identified based on retention times and comparison to authentic standards.

As has been demonstrated for folic acid, the high sensitivity of AMS analysis can reveal the formation of previously unidentified metabolites that are formed at levels below the limits of detection achievable with traditional techniques (Clifford *et al.*, 1998). A further feature is the capability to detect metabolites formed after low-dose exposures, using doses of labeled chemicals comparable to therapeutic or environmental levels. This methodology was applied in a study designed to assess the distribution of PhIP and its metabolites in lactating female rats and their pups after administration of a single dose of ^{14}C-PhIP, equivalent to an average human daily dose of this compound (Mauthe *et al.*, 1998). The detection of PhIP and PhIP metabolites in the breast milk, stomach contents, and liver tissue of the pups by AMS provided evidence that suckling pups are exposed to this carcinogen even at low doses. To further examine the types and concentration of metabolites excreted in breast milk, metabolites were extracted by adding methanol and centrifuging the milk samples to remove protein. The supernatant was evaporated to dryness, the residue dissolved in 0.1% trifluoroacetic acid, and subject to reverse-phase HPLC separation using a trifluoroacetic acid/acetonitrile mobile phase. This procedure achieved greater than 95% recovery of PhIP and its metabolites, based on the radiocarbon levels of samples measured before and after extraction. Fractions were collected at 1-min intervals throughout the run. These were then concentrated and redissolved in water/methanol before adding tributyrin and preparing for AMS analysis. Reconstruction of the HPLC chromatogram using the AMS data revealed the presence of three metabolite peaks in addition to the parent PhIP molecule (Fig. 6). These were identified by comparison to metabolites present in milk extracts from a rat treated with a high (10 mg/kg) dose of PhIP, which were fully characterized by HPLC-MS. This study demonstrates the importance of being able to investigate low-dose metabolism, because although the metabolite profile over the dose range examined was qualitatively similar, two glucuronide conjugates formed at high doses (10 mg/kg) of PhIP were not detectable at low doses.

As stated previously, other separation techniques can be used in conjunction with AMS. Binding of ^{14}C-labeled molecules to proteins has been quantified and specific protein targets identified, by combining polyacrylamide gel separations (one dimensional or two dimensional) with AMS detection (Vogel *et al.*, 2001; Williams *et al.*, 2002). Typically, gel bands are excised and after drying under vacuum can be directly analyzed, because the polyacrylamide, which is produced from petroleum-based chemicals, contains sufficient carbon (51%) to act as a carrier. The total amount of carrier carbon can be calculated if the volume and composition of the excised gel band is known.

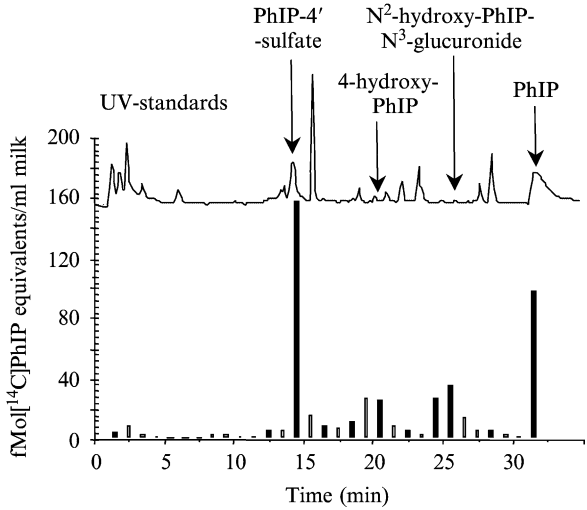

FIG. 6. High-performance liquid chromatography-accelerator mass spectrometry (HPLC-AMS) isotope chromatogram of ^{14}C-PhIP metabolites detected in breast milk from a female rat administered PhIP at 1000 ng/kg. The top chromatogram shows ultraviolet detection of metabolite standards.

AMS Detection of Chemically Modified DNA and Protein

AMS has demonstrated great value in determining whether chemicals bind to DNA or protein, forming covalent adducts. This is important because DNA adduct formation is considered an early initiating event in chemical-induced cancers. The presence of protein adducts, which may be formed at higher levels than DNA adducts, indicates a chemical is either itself reactive or can be converted to a reactive metabolite capable of binding to cellular macromolecules. Protein adducts can serve as surrogates for DNA adducts, giving a measure of an individual's level of exposure to the bioactive dose of a compound. Techniques used for detecting adducts must be very sensitive because adducts are usually formed at extremely low levels, particularly in humans exposed to environmental or therapeutic doses of carcinogens or drugs. AMS has been used to detect DNA adducts at levels of 1–10 adducts/10^{12} nucleotides, which is less than 1 modification/cell, following acute and chronic exposure to ^{14}C-labeled carcinogens (Turteltaub *et al.*, 1993). This is at least 100-fold lower than the next most sensitive detection method, the ^{32}P-postlabeling assay.

One of the first biomedical applications of AMS was to determine whether the compound MeIQx, which is formed in cooked meat, binds to

DNA in rodent tissues at low doses and whether the relationship between adduct formation and dose is linear (Turteltaub *et al.*, 1990). Since then, this technique has been used to answer similar questions for a number of chemical carcinogens in humans and animal models, including benzene (Mani *et al.*, 1999), trichloroethylene (Kautiainen *et al.*, 1997), and the breast cancer drugs tamoxifen and toremifene (Boocock *et al.*, 2002; Martin *et al.*, 2003; White *et al.*, 1997).

In this type of study, DNA and protein are isolated from tissues using standard protocols. For measurement of DNA adduct levels, tissues are lysed and digested using the enzymes proteinase K, RNase T_1, and RNase A. The lysate is loaded on to a Qiagen anion exchange column, and the protein and other material, including non-covalently bound labeled compounds and metabolites, are washed from the column, while the DNA is retained (Frantz *et al.*, 1995). The DNA is then eluted, precipitated, and washed with 70% ethanol before redissolving in a buffer appropriate for AMS analysis. It is important to demonstrate that all non-covalently bound compounds are removed by the isolation and purification procedure employed. For example, DNA could be enzymatically digested to nucleosides and separated by HPLC, ideally using a system that resolves adducted nucleosides from unmodified nucleosides and the free parent compound and metabolites (Mauthe *et al.*, 1999). This method should also remove other potential ^{14}C-labeled contaminants that may be present, such as residual adducted protein or peptides. AMS analysis of the collected fractions will indicate what percentage of the total ^{14}C or ^{3}H signal can be attributed to covalently bound adducts. Because this will be compound specific, performing this type of experiment will identify situations in which it may be necessary to incorporate additional DNA purification steps.

To isolate protein (and other acid precipitable macromolecules), homogenized tissues are lysed overnight and then centrifuged. Perchloric acid (70%) is added to the supernatant precipitating the protein. After centrifuging, the resulting protein pellet is washed with 5% perchloric acid, followed by several organic solvents (a 50% methanol solution and a 1:1 mixture of ether:ethanol) to extract any residual non-covalently bound ^{14}C-labeled compounds (Dingley *et al.*, 1999). The protein is then redissolved in 0.1 M of potassium hydroxide and aliquots of this solution are submitted for AMS analysis.

AMS can also be used to determine the ability of chemicals to bind to specific proteins, as was demonstrated by Dingley *et al.* (1999) in a study that reported PhIP binds to the blood proteins, hemoglobin, and albumin and to white blood cell DNA in humans administered a dietary relevant dose of [^{14}C]-PhIP. Adduct levels were monitored in five subjects over a

24 h period by taking blood samples (30 ml) at various times and separating into plasma, red blood cell, and buffy coat (containing white blood cells and platelets) fractions. Albumin and hemoglobin were isolated using standard protocols, with the addition of an initial dialysis step, and analyzed by AMS (Fig. 7). It is crucial when extracting albumin or hemoglobin that each sample of plasma or red blood cell lysate is first dialyzed extensively, in an individual beaker, for 48 h to remove non-covalently bound ^{14}C-labeled compounds and metabolites. This investigation demonstrated the value of AMS in providing information on adduct formation and

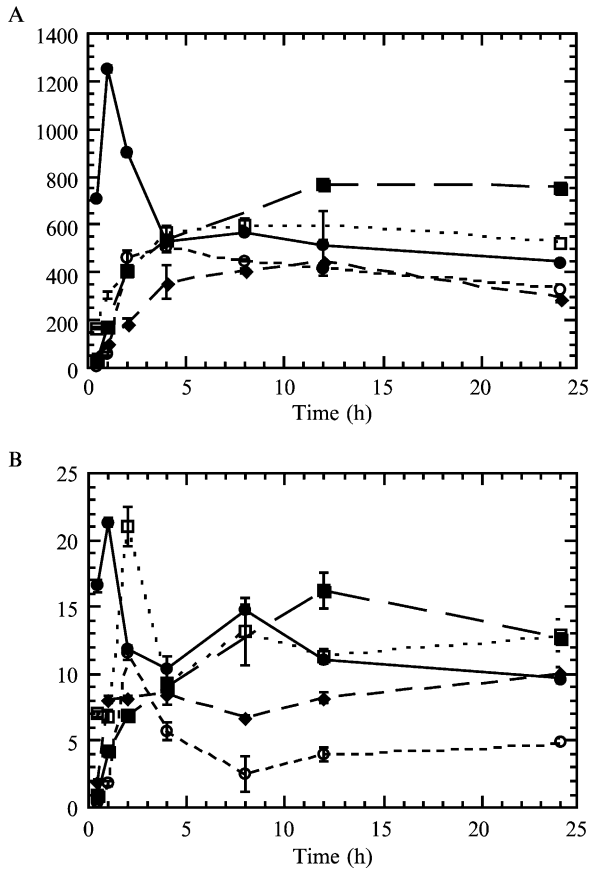

FIG. 7. Binding of PhIP to albumin (A) and hemoglobin (B) isolated from five human subjects administered a single dose of ^{14}C-PhIP.

kinetics in humans, which may be important in assessing an individual's susceptibility to specific carcinogens.

Protein samples, which are usually obtained in higher quantities than DNA, can normally be measured without the addition of carrier. Approximately 3 mg of protein will yield the required 1 mg of carbon, as will 3 mg of DNA when this amount is available, because both are composed of about 30% carbon, although this is dependent on tissue type and species. Smaller quantities can be analyzed (as little as 1 mg of DNA or protein), but there is greater potential for contamination and the sample may not produce sufficient graphite. In situations when the amount of tissue, and therefore, DNA is limited, as is often the case with human studies, the DNA is submitted with carrier. The amount of DNA analyzed in this way can be as little as 1 μg, although this will be influenced by the expected level of adduction, the compound specific activity, and availability. Material analyzed neat does not need to be accurately quantified, because the AMS measurement is a ratio, but for carrier-supplemented samples, the precise amount of DNA or protein must be determined. Consequently, when analyzing carrier-added samples, the precision and accuracy of the AMS data can be limited by the precision and accuracy of the methods used to quantify the mass of material. Alpha-particle energy loss measurement was developed as an accurate way to nondestructively quantify the mass of microgram quantities of macromolecules with high precision before analysis by AMS (Grant *et al.*, 2003).

The Future

AMS methodology has advanced substantially over the last 10 years, resulting in its now routine use in the measurement of ^{14}C and to a lesser extent ^{3}H in biological samples. The technique offers unique capabilities in studies where low amounts of radioisotope-labeled compounds must be quantified with high precision. These qualities have led to the increasing use of AMS in areas of nutrition, biomarker detection, human metabolism studies, and drug development. Methods for the analysis of additional isotopes are being developed.

Over the coming years, we anticipate AMS applications will expand and become more diverse as smaller cheaper instruments designed specifically for biologists are constructed and the technology becomes more accessible. Furthermore, automation of the sample-preparation process will increase sample throughput and could allow for analysis of smaller samples, all of which will help expand AMS into all areas of biological and environmental research.

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