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Accelerator Mass Spectrometry in Pharmaceutical Development

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Mass Spectrometry Handbook

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Accelerator Mass Spectrometry in Pharmaceutical Development

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Accelerator Mass Spectrometry in Pharmaceutical Development

I. Introduction to Biological Accelerator Mass Spectrometry

A. Overview of Biological Accelerator Mass Spectrometry

Accelerator mass spectrometry (AMS) is an analytical method for the measurement of rare, long-lived isotopes with extremely high sensitivity. In contrast to traditional liquid scintillation counting (LSC), which relies on nuclear decay events to quantify radioactive material, AMS counts atoms of a rare isotope independent of decay by measuring the mass ratio of the radioisotope of interest relative to a stable isotope of the element. AMS is currently the most sensitive technique available for measuring ^{14}C, with the ability to quantify labeled material over six orders of magnitude down to the attomole (10^{-18}) level (Brown et al, 2005; Brown et al, 2006; Turteltaub and Vogel, 2000; White et al., 2004). The exquisite sensitivity of AMS has enabled the use of human subjects in a number of biomedical tracer studies that would not otherwise be possible, since experiments can be designed to produce negligible radiological exposure to the experimental subjects without perturbing the normal biology of the system under consideration (Brown et al. 2006; Turteltaub and Vogel, 2000).

AMS was initially applied in isotope dating in the fields of archaeology and earth science, and more recently has been applied to the realm of experimental biology, including the areas of pharmacology and toxicology (Turteltaub and Vogel, 2000). Applications of AMS in the biological sciences include investigation of tissue turnover rates (Spalding et al., 2008; Bergmann et al., 2009), pharmacokinetic parameters of drugs (Boddy et al., 2007; Lappin et al., 2006), toxicants (Buchholz et al., 1999a), and nutrients (Buchholz et al., 1999b), including absorption, distribution, metabolism and excretion, as well as protein and DNA binding of drugs and reactive metabolites (Malfatti et al., 2006; Brown et al. 2007) and risk assessment (Creek et al., 1997; Dingley et al, 1999). The ability to administer and accurately measure extremely low

levels of a compound of interest using AMS is especially promising for application in the realm of pharmaceutical development. Tracer experiments using AMS to elucidate the disposition and metabolism of investigational compounds in Phase I studies can provide valuable data that will help determine whether an investigational compound warrants further development. AMS is also well-suited for determining molecular targets of a compound of interest in order to identify pharmacological sites of action or toxicological mechanisms. Subtherapeutic or "microdosing" experiments have made increasing use of AMS in recent years, allowing investigational compounds to be introduced into humans earlier than would be possible using more traditional techniques (Lappin et al., 2006; Sandhu et al., 2004). Because of the sensitive, quantitative, and precise nature of the technique, AMS can provide important information regarding the disposition and metabolism of the test compound, and could potentially become an important early screening tool in drug development. Some situations in which AMS may be the most appropriate measurement technique include:

- Compounds with low bioavailability
- Compounds with low systemic distribution
- Highly potent compounds
- Compounds created by difficult synthesis procedures where only limited quantities can be produced
- Experiments where a tracer must be measured for long periods of time (weeks or months).

B. Capabilities and Limitations of AMS

AMS is most suitable for the measurement of long-lived, low natural abundance radioisotopes. It is important that the radioisotope of interest have a low natural occurrence so

that the labeled compound can be discriminated from background levels, while maintaining a low dose of the labeled material. This precludes the use of stable isotopes such as ^{13}C, which is naturally present in relatively high abundance and comprises approximately 1% of naturally occurring carbon. In addition to ^{14}C, many other radioisotopes are suitable for measurement by AMS, but are generally of limited utility to the biological scientist. Biological AMS studies to date have made use of ^{3}H (Dingley et al., 2008), ^{41}Ca (Fitzgerald et al., 2005) and ^{10}Be (Chirarappa-Zucca et al., 2004).

AMS is unlike other forms of mass spectrometry in that the spectrometer is designed specifically for quantitation of the isotopes of a single element. AMS does not provide any structural information, and samples must be properly and adequately defined prior to AMS analysis in order to ensure that results are meaningful. Although a typical sample can be measured by AMS in a matter of a few minutes, the rate of sample preparation limits the throughput of the technique. Samples intended for AMS measurement must be converted to a form that is easily conductive in the ion source, and which does not allow for isotopic fractionation. Currently most spectrometers use solid graphite on a conductive core such as cobalt or iron (Vogel, et al., 2005; Ognibene et al., 2003). Thus, in order to measure a single HPLC trace, it may be necessary to prepare up to one hundred samples for AMS by graphitization (Buchholz et al., 2000a). Because of the sample preparation time, it can require several days between the time the samples were collected and the time when they are measured by AMS.

AMS is not suitable for all labeling experiments, particularly those in which sample material is plentiful and radiological dose is not an important consideration. Other techniques that may be considered in place of AMS include liquid scintillation counting (LSC), isotope ratio mass spectrometry using ^{13}C, ^{13}C-NMR, or in some case fluorescent or chemical tagging of the compound or molecule or interest. The level of sensitivity, importance of maintaining a low

radiological dose, number of samples, complexity of samples, and cost of analysis are major considerations in determining the appropriateness of using AMS.

C. ^{14}C Measurement by Accelerator Mass Spectrometry

Because most biological materials and drugs contain carbon, the majority of AMS measurements are performed with ^{14}C-labeled compounds. In a typical biological AMS study, a ^{14}C-labeled compound of interest can be routinely detected and quantified with 1-3% precision at levels ranging from approximately 10 pmol ^{14}C to 1 attomol (1×10^{-18} mole) of ^{14}C in samples containing one milligram of total carbon (Vogel and Love, 2005; Brown et al., 2005). AMS experiments in humans have used ^{14}C label ranges from 10 nCi/person to 50 µCi/person, depending on the duration and type of study being performed (Brown et al., 2005). The biological AMS instrument at Lawrence Livermore National Laboratory has measured over 70,000 samples during its operation over a period of nearly 10 years (Ognibene et al., 2004). Important performance characteristics of this instrument are listed in Table 1.

Table 1. Basic performance characteristics for the biological AMS instrument at Lawrence Livermore National Laboratory

Instrumental Performance	**Approximate Value**
Specificity (Background signal with no ^{14}C present)	2 parts per 10^{15}
Stability (Loss of ^{14}C during sample preparation)	CV < 3%
Linear Range of Measurement	10^{-2} – 10^{3} Modern
Instrument Carryover Between Samples	none
Reproducibility	< 5% error
Precision	2.5 % (Varies based on counting time)
Lower Limit of Quantitation	5 amol ^{14}C
Upper Limit of Quantitation	100 fmol ^{14}C
Signal Recovery	98%

Most AMS instruments measure samples in the solid state, although recent developments have allowed for sample measurement in the gas phase (McIntyre et al., 2009; Uhl et al., 2004; Flakaros et al., 2008). Measuring solid samples results in minimal experiment-to-experiment carryover of signal in the instrument, but also necessitates care in the preparation of samples for analysis. In order for biological material to be measured by AMS, it must first be converted to graphite. Optimal conversion of samples to graphite and measurement by AMS occurs with samples containing 0.5- 1 mg of carbon, although samples containing 0.3- 5 mg carbon may be successfully measured (Vogel and Love, 2005; Buchholz et al., 2000b) Samples containing smaller quantities of carbon can be measured by the addition of a known quantity of carrier carbon that is ^{14}C-deficient. The graphitization process consists of combustion of the biological sample to CO_2, cryogenic isolation of CO_2, and reduction to graphite in the presence of a cobalt or iron catalyst, as depicted in the Figure 1:

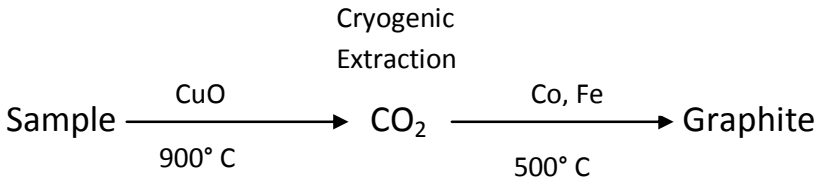

Figure 1. Overview of the sample graphitization process Sample material containing ^{14}C is combusted in the presence of CuO, CO_2 is cryogenically extracted, and CO_2 is reduced to graphite using a cobalt or iron catalyst.

The graphitization procedure has been described in greater detail elsewhere (Ognibene et al., 2002; Getachew et al., 2006; Vogel and Love, 2005).

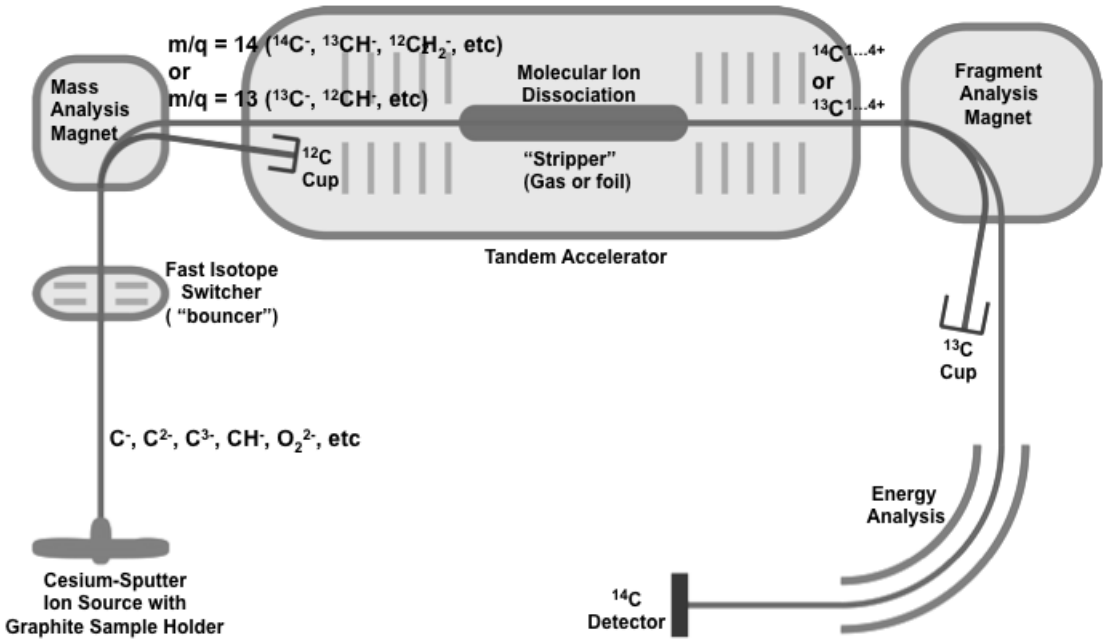

Figure 2: Schematic representation of the components of an accelerator mass spectrometer used to measure ^{14}C.

As depicted in Figure 2, a cesium sputter ion source is used to generate negative carbon ions. The ion beam is mass selected following passage through a magnet and introduced to the entrance of a tandem electrostatic accelerator. Interfering molecular isobars are destroyed in velocity-dependent collisions with either a diffuse argon gas or a thin carbon foil that also removes one or more electrons. The resultant positive ion beam is then further accelerated, followed by momentum and energy analysis. The rare ion (^{14}C) is counted using standard particle detection instrumentation. A change to the ion beam energy just after the ion source allows for the transmission of mass-13 ions through the first magnet and tandem accelerator with quantification of the $^{13}C^+$ ion current using an off-axis Faraday cup. This "bouncing" of the energy of the ion beam, occurs several times a second allowing for the near simultaneous measurement of the $^{14}C/^{13}C$ isotope ratio of a sample. Absolute quantification of the $^{14}C/C$ isotope ratio comes from comparing to the measured ratio of similarly prepared standards. This ratio is given in units of Fraction Modern.

One Modern is equal to:

- 1.180×10^{-12} $^{14}C/C$
- 13.56 mdpm/mg C
- 6.11 femtoCi/ mgC
- 97.89 amol/ mgC

II. Applications of Accelerator Mass Spectrometry in Drug Development

A. Basic Principles

Each biological AMS experiment is unique and must be designed on an individual basis. However, several basic principles can be applied in order to ensure that results are valid and informative. From the perspective of the investigator, it is absolutely essential to thoroughly understand what is being measured by AMS. This understanding can be achieved by considering three fundamental requirements for a successful AMS study. These requirements include:

(1) Absence of contamination in the sample,

(2) Appropriate amount of labeled compound, and

(3) Adequate sample definition.

Failure to satisfy one or more of these requirements can be very costly in time and resources, and may entirely negate the value of the AMS experiment. The absence of contaminating sources of ^{14}C is necessary to ensure that the measured ^{14}C is entirely due to the presence of the labeled compound. Contamination can result with laboratory background levels as low as a few femtocuries. The amount of labeled compound used must result in a sample that falls in the

range of 5 amol ^{14}C to 100 fmol ^{14}C in a milligram-sized sample Modern. If a sample contains more than 100 fmol ^{14}C (6.1 pCi/ mg C), the signal may exceed the linear range of the counting electronics and the detector may be damaged. Conversely, a sample that contains less than 5 amol ^{14}C will not produce adequate signal above background to be measured. Finally, the sample must be appropriately characterized by other methods such as HPLC prior to AMS in order to ensure that the correct and specific material of interest will be measured. Chemical purity and radiopurity of the starting material should also be well characterized, typically using HPLC/MS and decay counting. Careful attention to these principles allows the researcher to properly design experiments to obtain meaningful results.

1. Work Area and Chemistry Requirements

The work area for conducting biological AMS experiments and performing sample preparation procedures must be clean and free of sources of ^{14}C-radiolabeled material as well as sources of extraneous carbon. For AMS sample preparation, a laboratory that has no history of ^{14}C material use should be selected, as even fCi quantities of ^{14}C can contaminate experimental samples (Buchholz et al., 2000b, Zermeno et al., 2004). The area should be checked for ^{14}C background prior to beginning AMS experiments, and must be monitored regularly for contamination. LSC is not sufficiently sensitive to detect contamination at low levels that can interfere with correct AMS measurement. Surveys for radioactive contamination should be taken and sent for AMS analysis in any laboratory in which samples are routinely prepared for AMS. The surveying procedure is described in greater detail elsewhere (Buchholz et al. 2000b), and consists of the following steps:

1). Survey the area with a GM counter and survey as appropriate for LSC counting. If the GM counter or the LSC survey detects any contamination, the area cannot be used for AMS work without identification, and where possible, removal of the contamination.

2). If GM and LSC assays detect no contamination, proceed with collection of surveys for AMS measurement.

3). While wearing gloves, wet a glass fiber filter with isopropanol, and place this in a LSC vial as a blank.

4). Remove gloves and put on a new pair, wet a new glass fiber filter with isopropanol, and lightly rub over the area to be surveyed. This area is generally 100 cm^2.

5). Place the glass fiber filter in a LSC vial, cap, and label. Place only one survey in each vial. Repeat as needed until enough surveys are taken for the work area.

6). Submit filters for AMS analysis.

It is advisable to use disposable materials as much as possible to avoid the possibility of spreading contamination between samples. Samples should be handled using “sterile technique”. The area for preparation of the radiolabeled compound must be separate from the dosing and sample collection areas, and samples to be sent for AMS measurement must be stored separate from labeled material to prevent the possibility of contamination.

Chemicals to be used in AMS sample preparation and in stages of the experiments leading up to the AMS experiment must be free of interfering radioisotope. Samples of chemicals to be used should be checked to determine the background ^{14}C levels by scintillation counting and by AMS. Because AMS measures isotope ratios, it is likewise important to track all additions of carbon-containing compounds during the biological experiments and during sample preparation. Sodium-containing reagents should be avoided, as sodium can cause the quartz tubes used for graphitization to fail during the sample combustion step, resulting in loss of the sample. In many cases, it is possible to substitute potassium for sodium in reagents.

The major sources of potential error encountered in AMS are the addition of carbon or ^{14}C from sources that remain unaccounted for. This can be a result of contaminated solvents, glassware, laboratory equipment, or even other researchers (for example, airborne contamination). The use of carbon-containing solvents for extractions or separations should be carefully considered in the determination of the total carbon present in the sample. Miscalculations in dosing, or dosing of material that has an activity higher or lower than expected, can result in excessive or inadequate signal intensity from the AMS sample. Re-using glassware or pipettes can rapidly spread contamination among samples.

2. Dosing and Collection of Labeled Material

It is essential that the amount of ^{14}C label introduced into the experimental subject results in a measurable signal in the final sample, without exceeding the dynamic range of the instrument. In many cases, the amount of ^{14}C-labeled compound necessary for adequate recovery and detection of signal by AMS can be approximated using several calculations. It is always advisable to perform initial experiments to optimize dosing, recovery, and detection parameters prior to beginning the full-scale AMS study. Performing these calculations and initial experiments will greatly increase the probability of obtaining meaningful results in the AMS study.

In calculating the doses of labeled compound that should be administered in order to achieve a desired degree of labeling in the collected sample, it is helpful to know the bioavailability and volume of distribution of the compound under consideration. If these parameters are unknown, they can be determined using standard procedures or using AMS. The type and quantity of tissue or other material (e.g. urine, feces) to be sampled should also be considered in the dosing calculation. For an experiment with an injected or highly bioavailable

compound in which plasma will be collected and the labeled compound will be measured, the predicted optimal dosing level can be calculated as follows:

$$\text{Dose (in nCi)} = M_{Target} \times 6.11\ \text{fCi/mg C} \times V_{Distribution} \times C_{plasma}$$

Where

M_{Target} = Target fraction Modern in the collected sample, where $1 < M_{Target} < 100$

$V_{Distribution}$ = Apparent volume of distribution for the compound

C_{plasma} = Carbon content of plasma (mg C/ µL).

The dose typically ranges from 10 nCi- 50 µCi. In the case of a compound with low bioavailability, the dose must increase according to the equation:

$$\text{Dose (in nCi)} = M_{Target} \times 6.11\ \text{fCi/mg C} \times V_{Distribution} \times C_{plasma} \times (1/F)$$

Where

F = Bioavailability of the compound.

Predicted optimal doses can also be calculated for time points after administration of the labeled compound. In this case, the calculation is made based on the target fraction Modern for the final collection time point:

$$\text{Dose (in nCi)} = M_{Final} \times 6.11\ \text{fCi/mg C} \times V_{Distribution} \times C_{plasma} \times (1/F) \times e^{N}$$

Where

N = number of elimination half-lives at the time of final sample collection.

These equations can be used for other materials (e.g., urine) by substituting the carbon content and tissue or fluid volume, as appropriate. When the target dose has been calculated in nCi,

the quantity of labeled compound (e.g., nmol) can be calculated based on the specific activity of the compound (nCi/nmol):

Quantity (nmol) = Dose x Specific Activity.

The quantity of compound administered is typically in the range of 10-100 nmol.

For novel compounds where little is known about bioavailability, volume of distribution, and elimination half-life, it may be necessary to perform initial range-finding experiments starting with low doses and working upward prior to conducting full scale experiments. All samples with unknown levels of radioactivity should be quantified by scintillation counting prior to graphitization and AMS analysis. Any sample that can be measured by scintillation counting contains too much label to be measured by AMS.

Sample collection should be carried out using disposable materials, and care should be taken to avoid contamination or mixing of samples during collection, handling, and storage. Due to the possibility of introducing contamination, a separate set of pipettes should be used for dosing experiments and for preparing samples for AMS analysis. If pipettes are used for dosing, they should not be used for general lab work or for preparing samples for AMS submission. Positive displacement pipettes and filter tips should be used at all times in all stages of experimental work and sample preparation to avoid the possibility of label carry-over from one sample to the next. It is wise to always use new, disposable laboratory vessels and pipettes for all materials used in AMS experiments. This minimizes the potential for spread of contamination. For all experiments, control samples (pre-dose or undosed) must be generated in order to provide a ^{14}C baseline for each experiment. Samples should be collected individually and sealed. Dosing stocks should not be stored with AMS samples.

3. Sample Definition and Preparation

During the process of converting the sample material to graphite for AMS measurement, all chemical information contained in the original sample is lost. Thus, it is vitally important that the sample be adequately characterized prior to AMS analysis. This characterization will be specific for each type of sample, and may in many cases be as simple as collecting and preparing a selected tissue or organ (liver, lung, kidney, etc.). More complicated experiments may require additional separation and characterization techniques including mass spectrometry, HPLC, ELISA, immunoblotting, or other analytical techniques as needed to adequately separate and confirm the identity of the sample material.

It is essential to maintain a complete and accurate “carbon inventory” in order to produce meaningful data by AMS, since both total carbon and ^{14}C are measured to generate an isotope ratio. With some samples it may be impossible to achieve the desired carbon amount. In this case, it may be necessary to add "carrier carbon" to these ultra-low carbon mass samples. The primary source of carrier carbon now in use is tributyrin (glycerol tributanoate). This substance has the desirable characteristics of nonvolatility (bp >300°C), high carbon content, and low ^{14}C content. A typical addition of carrier carbon occurs by adding 1 or 2 µl of tributryin in capillary tubes to the sample. It is always advisable to analyze each new type of sample for carbon content prior to graphitization to ensure that a proper carbon inventory is maintained.

4. Sample Analysis and Interpreting the Results

AMS reports results in Modern, which is a defined unit of $^{14}C/C$ isotope concentration equal to 1.18×10^{-12} $^{14}C/C$. Conversion of this number to a meaningful result requires careful inventory of the carbon sources in the sample. For the simplest case in which a sample contains sufficient carbon as not to require the addition of carrier carbon, this conversion is facile. In many experiments, it is necessary to add carrier to make up the total amount of carbon necessary for the graphitization process to occur. In these experiments, the fraction

Modern and the carbon mass of both the sample and the carrier must be considered in the calculation. Correctly accounting for all sources of carbon in the analyzed sample is essential, as a low estimate of carbon will artificially increase the apparent quantity of tracer, and an overestimate of carbon will deflate the apparent amount of tracer present.

The measured ratio is a composite value that includes the total amount of ^{14}C and the total carbon present in the measured sample. It is necessary to know the quantity of carbon and ^{14}C in the sample tissue, as well as the amount of carbon due to the tracer, in order to solve for the ^{14}C due to the tracer. If the only sources of carbon in the measured sample are the tracer and the tissue, determining the ^{14}C due to the tracer proceeds as follows:

$$R_{Measured} = \frac{^{14}C_{measured}}{C_{measured}} = \frac{^{14}C_{tracer} + {}^{14}C_{tissue}}{C_{tracer} + C_{tissue}} \qquad (1)$$

In most cases, the carbon due to the tissue is much greater than the carbon due to the tracer, so that the tracer carbon can be neglected in the calculation:

$$R_{Measured} = \frac{^{14}C_{tracer} + {}^{14}C_{tissue}}{C_{tissue}} \qquad (2)$$

Because the measured fraction Modern is a composite of the contribution of the tissue and the contribution of the tracer, rearranging to solve for the tracer ^{14}C gives:

$$^{14}C_{tracer} = C_{tissue} \times (R_{Measured} - R_{Tissue}) \qquad (3)$$

where,

$$R_{tissue} = \frac{^{14}C_{tissue}}{C_{tissue}} \qquad (4)$$

If carrier carbon is also present, the general equation is as follows:

$$R_{Measured} = \frac{^{14}C_{tracer} + {}^{14}C_{tissue} + {}^{14}C_{carrier}}{C_{tracer} + C_{tissue} + C_{carrier}} \qquad (5)$$

In this case, the carrier carbon is usually much greater than the carbon due to the tissue or the tracer, allowing these quantities to be neglected in the calculation:

$$R_{Measured} = \frac{^{14}C_{tracer} + {}^{14}C_{tissue} + {}^{14}C_{carrier}}{C_{carrier}} \qquad (6)$$

Which becomes:

$$^{14}C_{tracer} = C_{carrier} \times (R_{Measured} - (R_{tissue} + R_{carrier}) \qquad (7)$$

Additional example calculations can be found in Section III.

B. Specific Applications of AMS

AMS excels at detecting very low levels of labeled compounds of interest, and has been applied to a variety of types of experiments applicable to drug development. The common feature of these experiments is the ability to trace the fate of a labeled compound in a biological system. This can include determination of pharmacokinetic parameters, biotransformation of the labeled compound, formation of covalent adducts with cellular macromolecules, drug-ligand

interactions, and tissue or sub-cellular localization of the compound of interest. Such experiments can provide valuable information about the pharmacological and toxicological characteristics of investigational compounds. AMS may likewise be used to investigate metabolism of endogenous, naturally-occurring compounds within a biological system without perturbing the normal physiology of the system. The types of experiments listed below are by no means exhaustive, but are intended to give the reader a sense of the potential value of AMS in the drug development process.

1. Pharmacokinetics

Several studies have compared the pharmacokinetic profiles of drugs using standard therapeutic doses and sub-therapeutic doses, commonly known as "microdosing" (Miyaji et al. 2009; Sandhu et al., 2004; Lappin et al., 2006). In these experiments, a sub-therapeutic level of a ^{14}C-labeled investigational compound is administered to animals or human volunteers, and the plasma levels of the compound are measured over time to determine the clearance rate and other pharmacokinetic parameters of the compound. These experiments are essentially the same as traditional "dose and measure" pharmacokinetic experiments, but the investigational compound can be administered and measured at much lower levels than are possible using standard techniques. This allows for human studies to be performed without the risk of harmful effects that could occur with the administration of higher doses. While the utility of such microdosing experiments as a replacement for standard pharmacokinetic techniques remains a matter of discussion, several reports indicate that microdosing may be useful as a screening tool for drug candidates (Lappin et al., 2006). An understanding of the pharmacokinetic parameters of a drug candidate may disqualify the compound for further investigation based on a poor pharmacokinetic profile, or may provide guidelines for predicting optimal doses within the therapeutic range.

In planning and conducting pharmacokinetics studies using AMS as a measurement technique, it is essential that an appropriate dose of the labeled material be administered to the test subject in order to allow accurate measurement at the selected time points. Assuming that the quantity of drug to be administered is significantly below the level required to elicit a pharmacological or toxic effect, dosing can be calculated based on the level of ^{14}C required to detect an adequate signal in the collected sample. If higher levels of drug are to be administered, the dose should be calculated based on the therapeutic dose range in addition to the radiological dose. The general steps involved in performing a pharmacokinetics study using AMS are as follows:

- Determine the specific activity of the ^{14}C-labeled compound of interest to be used in the pharmacokinetics study.
- Determine the mode(s) of administration of the compound of interest (injection, oral, dermal, subcutaneous, etc.). If appropriate, estimate the bioavailability of the compound.
- Determine the anticipated length of time the compound should be followed, the number of samples to be collected (blood, urine) and the sampling interval.
- Determine the minimum target fraction modern to be achieved in the sample (blood, urine) at the final time point. In most cases, this level should be at least 10% above 1 Modern in order to allow appropriate resolution.
- Calculate the initial dose based on the estimated number of biological half-lives of the compound to produce the desired ending fraction modern. Determine the total radiological exposure to be achieved using the calculated initial dose, assuming that all radioactive decay energy will be absorbed by the subject. Consult with a health

physicist to ensure that this level of exposure is acceptable based on current regulations and requirements. For purposes of assessing the radiological burden, a human can be assumed to have a normal ^{14}C level of 1 Modern, which for a 70 kg person corresponds to an energy deposit of 110 nJoules/hour, and assuming that the radiological dose is uniformly distributed throughout the body, this corresponds to 1.6 nSv/hour. (Vogel and Love, 2005).

- If no information about the compound's bioavailability and biological half life is available, calculate the initial dose based on the target highest amount of label to be present in the sample, and proceed to calculate the total radiological exposure.

- The initial samples collected should be measured by LSC to ensure that the calculations were correct and that the level of dosing was correct for AMS measurement.

2. Biotransformation and Conjugate Formation

Identification of metabolites of a drug candidate or other molecule of interest can be greatly facilitated by AMS. Tracing of a ^{14}C label allows unequivocal identification of compounds originating from the ^{14}C-labeled parent compound, including metabolites and degradation products, even if the structures of these compounds are not previously known. This type of experiment may be performed by collecting blood and urine and separating components by HPLC or other separation techniques, and identifying the parent compound as well as any metabolites or conjugates.

Appropriate separation techniques are essential for the identification of metabolites. Although the presence of label introduced as the parent compound unambiguously demonstrates the source of the label, it is important to ensure that the collected material is reasonably pure to allow further characterization of novel metabolites. In many instances, the

metabolites of a compound of interest may be unknown or incompletely characterized. A typical metabolism study consists of the following components:

- Characterize chemical purity, radiopurity, and specific activity of the ^{14}C-labeled compound of interest
- Administer the labeled material and collect samples (blood, urine, tissue, etc.)
- Perform mass balance to account for all labeled material
- Separate metabolites by standard techniques (HPLC, electrophoresis, etc.)
- Remove any solvents used in separations processes and analyze the sample fractions by AMS, adding carrier as necessary. In the case of HPLC fractions, the amount of carbon present in each sample is usually negligible compared to the amount of carrier carbon.
- Analysis occurs by comparison of detector peaks (for example, UV) with AMS results to identify fractions containing the parent compound, its metabolites, and conjugates (e.g., glutathione conjugates).

3. Protein and DNA Adduct Measurement for Determination of Reactive Metabolites

Incorporation of label into protein or DNA is a useful indicator of adduct formation or non-covalent binding of the moiety of interest. This approach has been used very successfully in the evaluation of known or potential carcinogens that exhibit DNA-binding activity (Brown et al., 2007; Coldwell et al., 2008; da Costa et al., 2003; Malfatti et al., 2006). This type of experiment may be particularly valuable in assessing the potential toxicity, mutagenicity, or carcinogenicity of a compound of interest. For example, AMS was used to detect DNA adducts in the colon after humans were exposed to a dietary-relevant dose of the cooked food carcinogen PhIP

(Malfatti et al., 2006). It may also be important to determine the quantitative fate of compounds that can form reactive intermediates capable of binding to cellular macromolecules, for example, compounds that can form immunogenic haptens or glutathione conjugates. Experiments of this type can also be designed to distinguish covalent and non-covalent interactions. As in the types of experiments described in previous sections, it is absolutely essential that the sample be thoroughly characterized and as pure as is reasonably achievable to ensure that results are not confounded by the presence of contaminating materials. These experiments include the following parameters:

- Determine the tissue to be sampled
- Calculate the dose of compound to administer and the quantity of tissue to collect
- Administer the dose and collect sample material
- Separate and quantify the biomolecule of interest (protein, DNA)
- Perform AMS measurement on the sample

4. Drug-ligand Binding and Subcellular Localization

Tracing the radiolabel into tissues, cells, and even subcellular compartments is possible using AMS (Beumer et al., 2007; Hah et al., 2007; Vuong et al., 2008). These experiments may be of great importance in determining the site of action and specific molecular target(s) of a drug or other compound of interest. Identifying the site of action or the molecular target of a drug candidate is very important in characterizing its biological activity and elucidating its mechanism of action. Such experiments could be a valuable screening tool to help in the selection of lead compounds for development as investigational drugs, or to indicate that a compound has undesirable binding or localization characteristics early in the drug development process. As with other types of experiments, the sample material must be thoroughly characterized prior to

AMS analysis. The steps involved in these types of experiments are similar to those described in the previous section, but may include cellular fractionation in addition to isolation of target biomolecules.

III. Example Calculation of Dosed Tissue Concentration

The conversion of a measured isotope ratio of a dosed tissue on the spectrometer to a biologically meaningful compound concentration in neat tissue is straightforward. The procedure below gives a concentration of molecular equivalents of parent compound in the tissue. The measured isotope ratio $R_{measured}$ can be expressed as shown previously in equation 1.

$$R_{measured} = \frac{^{14}C_{tracer} + ^{14}C_{tissue}}{C_{tracer} + C_{tissue}} \qquad (1)$$

where, $^{14}C_{tracer}$ is the amount of ^{14}C from the labeled compound,

$^{14}C_{tissue}$ is the endogenous level of ^{14}C in the tissue,

C_{tracer} is the amount of carbon from the labeled compound, and

C_{tissue} is the amount of carbon in the tissue.

Equation (1) is simplified in that $C_{tissue} >> C_{tracer}$ and can thus be neglected, as previously demonstrated in equation 4:

$$^{14}C_{tracer} = (R_{measured} - R_{tissue}) \times C_{tissue} \qquad (4)$$

where,

R_{tissue} = $^{14}C/C$ isotope ratio of the undosed tissue, obtained from the predosed control tissue.

Consider the results following example using a labeled compound (59 nCi, 58 Ci/mol) detected in plasma after an oral dose:

$R_{measured}$ = 2.50 Modern (1 Modern = 97.89 amol ^{14}C/mg C)

R_{tissue} = 1.08 Modern

C_{tissue} = 0.042 mg C/mg plasma

The excess ^{14}C per unit mass of tissue is calculated by application of equation 4:

$$^{14}C_{tracer}/\text{mg tissue} = (2.50\text{-}1.08) \times 0.042 \times 97.89$$

$$= 5.84 \text{ amol } ^{14}C/\text{mg plasma}$$

The excess ^{14}C/mass tissue can be converted to a compound concentration in the tissue, knowing that 1 ^{14}C/ C per molecule has a specific activity of 62.4 Ci/mol and that the density of plasma is about 1.02 g/ml:

$$\frac{\text{Moles trace compound}}{\text{volume tissue}} = \frac{\text{excess 14C}}{\text{gram tissue}} \times \frac{62.4}{\text{Sp. Act}} \times \rho_{plasma}$$

$$= 5.84 \times \frac{62.4}{58} \times 1.02 \times 10^3$$

$$= 6.41 \times 10^3 \text{ amol compound/ mL plasma}$$

$$= 6.41 \text{ fmol compound/ mL plasma}$$

IV. Summary

AMS is a powerful measurement technique offering exquisite sensitivity and precise quantitation of isotopically labeled compounds of interest. The primary use of AMS is in the measurement of very low levels of ^{14}C-labeled tracer compounds. Valuable information regarding absorption, metabolism, distribution, excretion, and toxicity of experimental compounds can be obtained through properly designed AMS experiments. The types of studies discussed include pharmacokinetics, biotransformation and conjugate formation, protein and DNA adduct measurement for determination of reactive metabolites, and drug-ligand binding and subcellular localization. AMS can also be applied to variations of these experiments, as well as additional types of experiments. This chapter has described the fundamentals of

biological AMS and provides a starting point for researchers interested in performing studies that involve AMS measurements. Researchers who have not previously conducted AMS experiments are strongly encouraged to consult with scientists experienced in AMS prior to beginning their own studies.

V. References

Bergmann O., Bhardwaj, R. D., Bernard, S., Zdunek, S., Barnabé-Heider, F., Walsh, S., Zupicich, J., Alkass, K., Buchholz, B. A., Druid, H., Jovinge, S., and Frisén, J. 2009. Evidence for cardiomyocyte renewal in humans. *Science* 324(5923):98-102.

Beumer, J. H., Garner, R. C., Cohen, M. B., Galbraith, S., Duncan, G. F., Griffin, T., Beijnen, J. H., and Schellens, J. H. M. 2007. Human mass balance study of the novel anticancer agent ixabepilone using accelerator mass spectrometry. *Invest New Drug* 25:327-334.

Boddy, A. V., Sludden, J., Griffin, M. J., Garner, C., Kendrick, J., Mistry, P., Dutreix, C., Newell, D. R., and O'Brien, S. G. 2007. Pharmacokinetic investigation of imatinib using accelerator mass spectrometry in patients with chronic myeloid leukemia. *Clin Cancer Res* 13(14):4164-4169.

Brown, K., Dingley, K. H., and Turtelatub, K. W. 2005. Accelerator Mass Spectrometry for Biomedical Research. *Method Enzymol* 402:423-443.

Brown, K., Tompkins, E. M., Boocock, D. J., Martin, E. A., Farmeer, P. B., Turteltaub, K. W., Ubick, E., Hemingway, D., Horner-Glister, E., and White, I. N. H. 2007. Tamoxifen forms DNA adducts in human colon after administration of a single [14C]-labeled therapeutic dose. *Cancer Res* 67(14):6995-7002.

Brown, K., Tompkins, E. M., and White, I. N. H. 2006. Applications of accelerator mass spectrometry for pharmacological and toxicological research. *Mass Spectrom Rev* 25:127-145.

Buchholz, B. A., Dueker, S. R., Lin, Y., Cliffors, A. J., and Vogel, J. S. 2000. Methods and applications of HPLC-AMS. *Nucl Instrum Meth B* 172:910-914.

Buchholz, B. A., Freeman, S. P. H. T., Haack, K. W., and Vogel, J. S. 2000. Tips and traps in the ^{14}C bio-AMS preparation laboratory. *Nucl Instrum Meth B* 172:404-408.

Buchholz, B. A., Fultz, E., Haack, K. W., Vogel, J. S., Gilman, s. D., gee, S. J., Hammock, B. D., Hui,X., Wester, R. C., and Maibach, H. I. 1999. HPLC-accelerator MS measurement of atrazine metabolites in human urine after dermal exposures. *Anal Chem* 71(16):3519-3525.

Buchholz, B. A., Arjomand, A., Dueker, S. R., Schneider, P. D., Clifford, A. J., and Vogel, J. S. 1999. Intrinsic erythrocyte labeling and attomole pharmacokinetic tracing of ^{14}C-labeled folic acid with accelerator mass spectrometry. *Anal Biochem* 269:348-352.

Chiarappa-Zucca M. L., Finkel, R. C., Martinelli, R. E., McAninch, J. E., Nelson, D. O., and Turteltaub, K. W. 2004. Measurement of beryllium in biological samples by accelerator mass spectrometry: applications for studying chronic beryllium disease. *Chem Res Toxicol* 17(12):1614-20.

Coldwell, K. E., Cutts, S. M., Ognibene, T. J., Henderson, P. T., and Philips, D. R. Detection of adiramycin-DNA adducts by accelerator mass spectrometry at clinically relevant adriamycin concentrations. 2008. *Nucleic Acids Res* 36(16):1-10.

Creek, M. R., Mani, C., Vogel, J.S., and Turteltaub, K. W. 1997. Tissue distribution and macromolecular binding of extremely low doses of [14C]-benzene in B6C3F1 mice. *Carcinogenesis* 18(12):2421-7.

da Costa, G. G., Marques, M. M., Beland, F. A., Freeman, J. P., Churchwell, M. I., AND Doerge, D. R. 2003. Quantification of Tamoxifen DNA adducts using on-line sample preparation and HPLC-electrospray ionization tandem mass spectrometry. *Chem Res Toxicol* 16:357-366.

Dingley K.H., Curtis, K. D., Nowell, S., Felton, J. S., Lang, N.P., and Turteltaub, K. W. 1999. DNA and protein adduct formation in the colon and blood of humans after exposure to a dietary-relevant dose of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine. *Cancer Epidemiol Biomarkers Prev.* 8(6):507-12.

Dingley, K. H., Roberts, M. L., Velsko, C. A., and Turteltaub, K.W. 1998. Attomole detection of 3H in biological samples using accelerator mass spectrometry: application in low-dose, dual-isotope tracer studies in conjunction with 14C accelerator mass spectrometry. *Chem Res Toxicol* 11(10):1217-22.

Fitzgerald, R.L., Hillegonds, D.J., Burton, D.W., Griffin, T.L., Mullaney, S., Vogel, J.S., Deftos, L.J., and Herold, D. A. 2005. ^{41}Ca and accelerator mass spectrometry to monitor calcium metabolism in end stage renal disease patients. *Clin Chem.* 51(11):2095-102.

Flarakos, J., Liberman, R, G., Tannenbaum, S. R., and Skipper, P. L. 2008. Integration of continuous-flow accelerator mass spectrometry with chromatography and mass-selective detection. *Anal Chem* 80(13): 5079-5085.

Getachew, G., Kim, S. H., Burri, B. J., Kelly, P. B., Haack, K. W., Ognibene, T. J., Buchholz, B. A., Vogel, J. S., Modrow, J., and Clifford, A. J. 2006. How to convert biological carbon into graphite for AMS. *Radiocarbon* 48(3):325-336.

Lappin, G., Kuhnz, W., Jochemsen, R., Kneer, J., Chaudhary, A., Oosterhuis, B., Drijfhout, W. J., Rowland, M., and Garner, R. C. 2006. Use of microdosing to predict pharmacokinetics at the therapeutic dose: Experience with 5 drugs. *Clin Pharmacol Ther* 80(3):203-215.

Malfatti, M. A., Dingley, K. H., Nowell-Kadlubar, S., Ubick, E. A., Mulakken, N., Nelson, D., Lang, N. P., Felton, J. S., and Turteltaub, K. W. 2006. The urinary metabolite profile of the dietary carcinogen 2-aminon-1-methyl-6-phenylimidazo[4,5-b]pyridine is predictive of colon DNA adducts after a low-dose exposure in humans. *Cancer Res* 66(21):10541-10547.

McIntyre, C. P., Sylva, S. P., and Roberts, M. L. 2009. Gas chromatograph-combustion system for ^{14}C-accelerator mass spectrometry. *Anal Chem* 81(15):6422-6428.

Miyaji, Y., Ishizuka, T., Kawai, K., Hamabe, Y., Miyaoka, T., Oh-Hara, T., Ikeda, T., and Kurihara, A. 2009. Use of An Intravenous Microdose of ^{14}C-labeled drug and Accelerator Mass Spectrometry to measure Absolute Oral Bioavailability in Dogs; Cross-comparison of Assay Methods by Accelerator Mass Spectrometry and Liquid Chromatography-Tandem Mass Spectrometry. *Drug Metab Pharmacok* 24(2):130-138.

Ognibene, T. J., Bench, G., Brown, T. A., and Vogel, J. S. 2004. The LLNL accelerator mass spectrometry system for biochemical ^{14}C-measurements. *Nucl Instrum Meth B* 223-224: 12-15.

Ognibene, T. J., Bench, G., and Vogel, J. S. 2003. A high-throughput method for the conversion of CO_2 obtained from biochemical samples to graphite in septa-sealed vials for quantification of ^{14}C via accelerator mass spectrometry. *Anal Chem* 75:2192-2196.

Sandhu, P., Vogel, J. S., Rose, M. J., Ubick, E. A., Brunner, J. E., Wallace, M. A., Adelsberger, J. K., Baker, M. P., Henderson, P. T., Pearson, P. G., and Baillie, T. A. 2004. Evaluation of microdosing strategies for studies in preclinical drug development: demonstration of linear pharmacokinetics in dogs of a nucleoside analog over a 50-fold dose range. *Drug Metab Dispos* 32:1254-1259.

Spalding, K. L., Arner, E., Westermark, P. O., Bernard, S., Buchholz, B.A., Bergmann, O., Blomqvist, L., Hoffstedt, J., Näslund, E., Britton, T., Concha, H., Hassan, M., Rydén, M., Frisén, J., and Arner, P. 2008. Dynamics of fat cell turnover in humans. *Nature.* 453(7196):783-7.

Turteltaub, K. W. and Vogel, J. S. 2000. Bioanalytical applications of accelerator mass spectrometry form pharmaceutical research. *Curr Pharm Design* 6:991-1007.

Uhl, T., Kretschmer, W., Luppold, W., and Scharf, A. 2004. "Direct Coupling of an Elemental Analyzer and a Hybrid Ion Source for AMS Measurements." *Radiocarbon* 46(1): 65-75.

Vogel, J. S. and Love, A. H. 2005. Quantitating isotopic molecular labels with accelerator mass spectrometry. *Method Enzymol* 402:402-422.

Vuong, L. T., Ruckle, J. L., Blood, A. B., Reid, M. J., Wasnich, R. D., Synal, H. and Dueker, S. R. 2008. Use of accelerator mass spectrometry to measure the pharmacokinetics and peripheral blood mononuclear cell concentrations of zidovudine. *J Pharm Sci* 97(7):2833-2843.

White, I. N. H., and Brown, K. 2004. Techniques: The application of accelerator mass spectrometry to pharmacology and toxicology. *Trends Pharmacol Sci* 25(8):442-447.

Zermeno, P., Kurdyla, D. K., Buchholz, B. A., Heller, S. J., Kashgarian, M., and Frantz, B. R. 2004. Prevention and removal of elevated radiocarbon contamination in the LLNL/CAMS natural radiocarbon sample preparation laboratory. *Nucl Instrum Meth B* 223-334:293-297.