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Quantifying exploratory low dose compounds in humans with AMS

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Abstract

Accelerator Mass Spectrometry is an established technology whose essentiality extends beyond simply a better detector for radiolabeled molecules. Attomole sensitivity reduces radioisotope exposures in clinical subjects to the point that no population need be excluded from clinical study. Insights in human physiochemistry are enabled by the quantitative recovery of simplified AMS processes that provide biological concentrations of all labeled metabolites and total compound related material at non-saturating levels. In this paper, we review some of the exploratory applications of AMS ^{14}C in toxicological, nutritional, and pharmacological research. This body of research addresses the human physiochemistry of important compounds in their own right, but also serves as examples of the analytical methods and clinical practices that are available for studying low dose physiochemistry of candidate therapeutic compounds, helping to broaden the knowledge base of AMS application in pharmaceutical research.

Keywords

Microdosing; Accelerator Mass Spectrometry; AMS; Metabolism; Microdose; Mass Balance; Nutrition; Physiochemistry; MIST; Microtracing; Absolute Bioavailability; Microtracer

2. Introduction

Accelerator Mass Spectrometry (AMS) is a form of isotope ratio mass spectrometry primarily developed in the late 1970's for radiocarbon dating at part-per-trillion to quadrillion isotopic ratios ($^{14}\text{C}/\text{C}$) to obtain precise ^{14}C carbon dates on smaller and older samples in archaeological and earth science. One or more stable carbon isotopes are accelerated sequentially or simultaneously and quantified for a fully normalized Isotope Ratio Mass Spectrometric analysis after the collision cell (Figure 1). The tracer isotope (^{14}C) is quantified as counted ions per the integrated ion currents of one or more stable isotopes. This ratio corrects the counted ^{14}C for changes in ion source intensity and emission (spreading angle off axis) and any variations in efficiency and overall transmission caused by changes in the amount and distribution of the gas or solid collision material. Core concepts of ^{14}C -AMS are summarized: negative ion production to eliminate the atomic isobar; high energy collisions to eliminate molecular isobars; ion identification of single atoms for specificity of response; and the normalizing ratio to stable carbon isotopes with

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equivalent ion pathways to eliminate quantitative effects from variable ion emission and transmission [1]. Kutschera provides a review of the many isotopes and applications that have benefited from the high sensitivity [2]. Over the past several decades AMS has also become an important bioanalytical tool for specific molecular pharmacokinetics and metabolism independent of chemical dose size.

Although the possibility for AMS to biomedical research was suggested shortly after its development, its use was not explored in earnest until the late 1980's, pioneered by efforts at the Lawrence Livermore National Lab [1,3-7]. Some of the earliest AMS applications laid the foundation for pharmaceutical and expanded research into biomedical by quantifying concentrations of metabolites in humans, primarily activated metabolites of environmental compounds that form genotoxic macromolecular adducts [8-11], agricultural chemicals that chronically expose workers at low levels [12-13], and vitamins at physiologically relevant doses [14-16]. Prior to AMS, such metabolites and their molecular interactions were only quantified at well above relevant biological concentrations due to the lack of analytical sensitivity.

^{14}C -tagged tracers have a rich history of use in the biological sciences [17], although the inefficient detection of ^{14}C by decay counting limited the extent and type of application (Less than 10 part-per-billion of the ^{14}C content in a sample is quantified in a typical decay measurement over a reasonable time-dependent acquisition period). The radiation emitted by ^{14}C is relatively benign and most compounds traced with a ^{14}C isotopic label are excreted in a few days, encouraging many human volunteers to generously accept the small radiation risks in order to promote biomedical science. Still, concerns about administration of ionizing radiation in excess of naturally occurring levels, uncertainty of the true human disposition, and the regulatory overhead associated with the transport, handling, and disposal of radioisotopes has stifled clinical use of the specificity for tracing and quantifying the fates of compounds to controlled clinical trials. Radiolabeled tracer studies for wider biochemical research and diagnostic programs were often more restricted. For example, micronutrient metabolism was initially discovered through key high radiation studies in often terminally ill volunteers or prisoners [18-19]. These studies formed the underpinnings of vitamin requirements for the general population and established aspects of fundamental human metabolism, but detailed radioisotope studies to understand the metabolic peculiarities of specific populations such as expectant/lactating women, children, or non-terminal diseased patients remain underrepresented in the literature. Thus the broader societal benefits of radiotracer research arise from the low sensitivity of the traditional detection technology. Despite radiation concerns, ^{14}C -tracer studies remain the most direct and definitive path to understanding a compound's physiochemistry, more specifically it's Absorption, Distribution, Metabolism and Elimination (ADME) profile, which can reveal potential "chemical" risks that arise from active metabolite production or off-target accumulation of the compound. Thus, ADME studies using 100 to 200 μCi -sized doses are a mainstay of drug development because the "chemical" risks of new compounds potentially outweigh the "radioactive" exposure risk. None the less, policies are in place to ensure that exposure to ionizing radiation is as low as Reasonably Achievable" (ALARA) or Practical (ALARP) [20-21]. AMS sensitivity for quantifying ^{14}C markedly reduces radioactive exposures to clinical volunteers and laboratory staff, supporting new definitions of ALARA in molecular tracer studies.

AMS was specifically developed to measure less than the natural levels of radiocarbon present at 100 amol ^{14}C per mg of carbon [22]. The high precision inherent to Isotope Ratio measurement provides limits of detection of only a few percent above this natural background for attomole detection of the isotope label in only 25 μl of plasma (approximately 1 mg of carbon) [23]. Even lower levels of quantification are achieved when

artificial baselines are applied using carbon carriers for chemical isolates that remove the natural ^{14}C background, such as liquid chromatography fractions [13]. The sensitivity of AMS permitted tracing the kinetics of ^{14}C -labeled compounds using amounts that are equivalent to, or a small factor of, a person's natural ^{14}C content (100 nCi, 4 kBq in a 70 kg person). This led to the concept of human microdosing, where the metabolism and pharmacokinetics (PK) of a drug are explored in low dose formats prior to committing to the expense of clinical Phase 1-enabling activities [24-25]. Microdosing as defined in the several regulatory guidance documents issued over the last decade, is human testing of what often amounts to 100 μg or less of a novel drug candidate under an abbreviated toxicological safety package. The abbreviated non-clinical safety package offers the promise of relatively "inexpensive" and "early" human data as being the final arbiter in candidate selection prior to initiation of standard Phase 1 programs. Microdosing equally includes Positron Emission Technologies (PET) studies but these areas of application are addressed elsewhere in this special issue. The first guidance to be issued by the EMEA came in 2004 and set the stage for expanded FDA guidance document in 2007 on exploratory IND applications that encompassed both microdosing proper and options for doses that would have limited therapeutic effects without challenging the maximally tolerated dose [26-27]. Both documents were agnostic to the mode of detection for PK determinations; modern LC/MS technologies often have sufficient sensitivity to 100 μg oral doses when volume of distributions and metabolism are not extensive and metabolism information is not a priority [28] [29]. AMS however is the instrument platform that can routinely quantify femtogram to picogram per mL concentrations without the requirement for calibrating reference material. The less appreciated advantage of the radiocarbon-AMS-microdosing triad is the ability to deliver ADME information including metabolism, routes of excretion, tissues sequestrations and macromolecular binding information from the specificity of radiolabel. When a ^{14}C -microdose is blended with a normal nonradioactive dose, sometimes by two different routes, the terms "microtracing" and "light label" are applied. These studies are conducted for metabolite discovery in early phase investigations, absolute bioavailability in a single test, and low radiation mass balance [30-31].

A harmonized ICH guidance (ICH Topic M 3 R2; Non-Clinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceutical) was put into place in 2009 which builds and extends the concepts for microdosing and exploratory clinical trials. Five "low dose" approaches were outlined which, significantly, encompass single microdose tests of $\leq 100 \mu\text{g}$ (or $1/100^{\text{th}}$ of the anticipated therapeutic dose) and options for repeat dosing designs, not to exceed 500 μg total chemical exposure in ≤ 5 dose administrations [32]. The possibility for iterative testing will markedly enhance the value of low dose testing through expansion of the multiple variables (formulation modification, i.v. vs. oral AUC, co-administration studies with inhibitors of drug metabolizing enzymes, meal effects, etc.) that can be interrogated in a suite of tests.

In this review, we show some of the exploratory applications of high sensitivity ^{14}C tracing at low chemical exposures that were made possible with AMS in toxicological, nutritional, and pharmacological research. We emphasize in this evaluation the body of research that reveals the human physiochemistry of important compounds, but also serves as examples of the analytical methods and clinical practices that are available for studying human physiochemistry of candidate therapeutic compounds, helping to broaden the knowledge base of AMS application in pharmaceutical research. Physiochemistry here encompasses both the local biochemistry of the compound under study along with the biochemical responses of physiological functions to the compound. Since the goal of therapeutic compounds is to effect specific physiochemical responses in humans, a greater understanding of complete physiochemical behavior of a compound directly in human volunteers provides not only the coarse integrated ADME data typical of pharmaceutical

development, but also new insights into specific and synergistic effects of enzymatic, binding, cellular, and membrane interactions of the compound. Such detail has seldom been available prior to AMS quantitation because of restrictions on chemical and/or radiation risk, limitations on sample size (CNS, alveoli, cells, etc.), and, finally, the lack of sensitivity and specificity to reliably attribute metabolic or transformation products to the dosed chemical.

It is noteworthy that AMS is primarily applied to quantifying tracer fate in complete living systems such as animals or humans, although the sensitivity of the method also allows quantification of very rare events or low concentration products in isolated systems such as cell cultures (e.g. Quantitation of NAD⁺ biosynthesis from the salvage pathway in *Saccharomyces cerevisiae*) [33]. There are many reviews of the evolution and application of AMS to biomedical research for both intact and isolate systems and the reader is referred to these excellent works [1,3,22-23,31,34-38]. The intent of the present review is to complement the existing body of research by touching on some of the lesser appreciated applications in low dose clinical studies. We do not directly review developments in the quantitative PK predictive power of microdoses as applied to candidate selection though this is a topic of high importance and relevance to any low dose publication. Forthcoming release of data from the on going validation (EUMAPP) trials will be should be sought by the reader as they become available [39-40].

3. Nutritional studies

Nutrient metabolism has particularly advanced under the use of AMS for sensitive isotopic tracing. Understanding the metabolism of vitamins, pro-vitamins and other bioactive phytochemicals is analytical challenging. They are required at trace levels and are often metabolized to multiple forms that are under continuous exchange with inputs from the diet as well as internal tissue and organ reservoirs. A radio-isotopic label assures that all circulating forms are detected and quantified so long as the label remains in association with the analyte of interest. The exploratory chemical doses must be commensurate with normally healthy intake to avoid toxic response to potent compounds (e.g. vitamin A) and to trace the natural metabolism at non-saturating levels. The distinguishable dosed compound is then available for quantifying effects of unlabeled interacting doses and effects.

3.1 Absorption of a lipophilic prodrug: β -Carotene

β -Carotene is a lipophilic plant pigment whose bioconversion to vitamin A active compounds within the intestine is the global mechanism for satisfying human and animal vitamin A needs [41]. The enzymatic capacity to perform this cleavage reaction is variable among people and metabolic differences among preclinical species render animal models of uncertain relevance [42]. For instance, felines cannot derive vitamin A from β -carotene whereas rats are hyper-metabolizers and do not absorb intact β -carotene. A series of experiments on the effects of diet and physiological status in healthy volunteers brought new understandings to β -carotene metabolism. These experiments are instructive as to how “microdosed” compounds review the underlying transport mechanisms, quantify metabolism, and guide delivery strategies of compounds transported and distributed by the lymphatic and lipoprotein systems.

A first AMS long-term kinetic study was conducted in a male subject receiving a 200 nCi (306 μ g) oral dose in a lipid suspension. Frequent blood collections taken during the absorptive phase were processed by HPLC to speciate β -Carotene and two major metabolites, retinyl esters and the alcohol form of vitamin A, retinol [15]. Total feces and urine voids were collected for >10 days post dose to assess routes of elimination and unabsorbed dose. Lymphatic instead of portal absorption was expected from prior literature and confirmed by subsequent plasma fractionation studies [43]. Concentration vs. time

course plots (Figure 1) indicated absorptive and distributive phases. The first absorptive phase (first peak) for these compounds obeyed a Gaussian function in time, which is characteristic of a facilitated absorption process over that of a passive concentration gradient, with the exchange rate both into and out of the plasma compartment approximately equal [3]. The remarkable congruency in early kinetic plot shapes among β -carotene and retinyl esters demonstrates a shared transport pathway between the dosed compound and its metabolite. The subsequent concentration peaks (post 10 hr) were attributed to a combination of multiple intestinal inputs and intra-plasma transfer of these components to plasma lipoprotein components, while the unusually linear rise in retinol levels was attributed to the controlled, post-absorptive secretion of hydrolyzed retinyl esters from hepatic tissue into the plasma in association with a specific binding protein. Quantifiable isotope signal in β -carotene and retinol extended for 7-months days post dosing, consistent with exchange between lipoprotein components in the plasma compartment and replenishment from tissue reservoirs.

The global importance of β -carotene relates to its ability to deliver bioactive retinoids to organs and tissues. Area-under-the-Curve (AUC) analysis for the β -carotene relative to retinyl esters in the early absorptive phase, adjusted for fecal losses during absorption lead to the conclusion that 25% of the theoretical vitamin A value was available from the single oral dose that was presumably non-saturating and well within typical dietary intake levels from a single meal. The quantitative precursor product relationship between these chemicals would not be obtainable from less sensitive stable isotopic methods without supra-pharmacological dosing which would cast doubt upon relevancy of the information.

3.2. Physiologic Status

Subsequent studies indicated that absorption proceeds even in when β -carotene is given with a low fat diet suggesting an important role for endogenous fat (bile acids) in the absorption process [43]. The role of nutritional status was further investigated in a longitudinal test-retest study (iterative testing format) where absorption and retention given equivalent 400 ng oral [^{14}C] β -carotene doses was assessed before and after 20 days of daily vitamin A supplementation (Figure 4; plasma metabolite data not shown) [44]. Urinary and fecal losses for total isotope were monitored at least 15 days. The results demonstrated that supplementation even in these nutritionally replete volunteers raised total absorption and body retention (body burden) of the administered dose as determined by output of isotope in the urine and feces. This surprising effect was attributed to an improvement in gut health and more efficient and prepared lymphatic transport mechanism due to supplementation.

The above data demonstrate the interpretive potential of high temporal resolution blood data supported by less detailed excretion information. The high data density provides the basis for interpretation of the physiochemistry ultimately governing disposition. Similar ADME information will prove useful in formulation development for lipophilic drugs and prodrug strategies that harness lymphatic absorption. In a microdose format under recent ICH M3 guidelines [32], iterative dosing schedules where the subjects serve as their own controls will reduce reliance on animal models while protecting the subjects from chemical and radiological harm.

3.3 Implications in Early Drug Development

Understanding and quantifying bioavailability in terms of intact parent and active metabolites within the lymphatic transport system was the objective of the β -carotene studies; these same concerns apply to pro-drugs under early development. Lymphatic transport and delivery as a prodrug are two strategic options that avoid first pass hepatic metabolism and alter clearance and deposition [45-46]. Prodrug approaches that enhance

lymphatic drug transport via the covalent coupling of drugs to lipid moieties including modifications with fatty acid, diglyceride or phosphoglyceride to obtain sufficient lipophilic character to access the fat transport system while not being diverted into the portal system (See Figure 5). Current studies directed towards understanding the many factors that determine abstraction into lymphatic transporter are conducted using animal and in vitro models [47-48] with uncertain predictability. The possibility for low dose iterative testing using in human presents an attractive economic and scientifically relevant solution when conducted under the microdose regulatory framework.

Low dose data also present cases when compartmental models must be invoked as the molecules kinetics are being governed by associations with endogenous processes and not governed by rapid mixing and partitioning. Low dose microdosing was initially touted as a means of pharmacokinetic prediction of the therapeutic dose with the intent to select the candidate with the optimal pharmacokinetics for continued development. While this remains an important objective, the value of low dose data for understanding the role of transporters, enzyme absorption and distribution should not be underestimated.

One pharmaceutical example is warfarin, which was one of the test drugs in the CREAM microdose validation studies of 5 compounds [25,49-51] (see Figure 6). The multiple decreases and resuspensions of the microdosed drug (open squares in plot) over time is potentially interpreted by associations with transporters and influx from tissues reservoirs. The authors hypothesized a low-capacity, high-affinity warfarin binding site was within the liver, vitamin K 2, 3-epoxide reductase, which may also be the therapeutic target [52]. The evaluation was listed as a “failure” in that the low dose kinetics did not scale to the high dose profile. An alternate view is it was not a failure, but a successful study of the underlying physiochemistry.

Exploratory microdosing thus provides the option for rapid iterative testing for pro-drug development, either for directing to lymphatic or more commonly portal absorption, often using simple ^{14}C bioanalysis that does not require compound specific method development. Inclusion of short-term urine and fecal provide a complete view of the drug disposition under various test parameters. Microdosing has been used to quantify delivery of pro-drug compounds in at least one published study [53], as well as for confirmation of in vivo production of bioactive and toxic metabolites identified in animal tests. These data and analyses ultimately strengthen the development in vitro and *in silico* PBPK models and thus their value will extend beyond any single compound study, but augment current prediction tools [40] [29]

4. Targeting drug: Cellular incorporation

AMS was early applied in bioanalysis to determine the turnover time of elastin in cadaver lung tissues [54]. This approach was recently expanded to determining the regenerative capability of specific cells in the nervous system [55], the insulin producing cells [56], and fat stores [57], by quantifying the amount of “bomb pulse” ^{14}C due to the atmospheric nuclear tests in the isolated DNA of these cells. Target binding confirmation is one objective of exploratory studies. Typically the domain of PET designs, AMS sensitivity enables quantitative analysis of small tissue biopsies following microdose chemical dose. The current body of *in vivo* human literature is limited; one published example of cellular uptake is found in folates studies. Folate was under investigation due to its central importance in one carbon metabolism to support hematopoiesis and suppressing neural tube birth defects in pregnant women [58]. Earlier studies had depended on ailing elderly male volunteers. The AMS-quantified study reduced the ^{14}C label to the point (35 μg ; 3.7 kBq; 100 nCi) that child-bearing-aged women (mean age = 24 yo) could be included in a mass balance

pharmacokinetic analysis of this highly retained and recirculated nutrient [59]. Following oral dosing, labeled folates incorporated into red blood cells (RBC) during a specific stage of hematopoiesis (more appropriately erythropoiesis), labeling a cohort of RBCs that passed through that stage during the day after the ^{14}C -folate dosing. These labeled RBC provided probes of the maturation time of the RBC after incorporation and then revealed details of lifetime and cellular retention of trapped folates in the RBCs. Mean lifetimes for the cell population were consistent with expected lifetimes in healthy normal populations (Figure 7). Additionally, the data pointed to large differences in individual uptake (bioavailability) balanced by very high biliary recycling that proved 24 times greater than previous estimates. This study demonstrated that accurately detailed kinetic profiles of very low dose tracer compounds can be used to demonstrate and quantify large reservoirs and retention paths within humans while quantifying specific cellular uptake within clinically available cell cohorts.

The capability of AMS to trace an administered compound in cellular targets is particularly relevant to anti-retroviral therapies where the concentration of cellularly retained drug is correlative with efficacy. Non-nucleoside reverse transcriptase inhibitors (NNRTIs) of human immunodeficiency virus type-1 (HIV-1) can be discovered within multiple chemical classes, and exploratory microdose trials are one strategy to select those having appropriate target coverage with the goal of improving on the currently available treatments. A microdose was administered intravenously and the ADME profiles recorded. The uptake and retention of total drug related material in isolated peripheral blood mononuclear cells (PBMCs) was quantified following isolation of the cells from ~ 2.5 mL of whole blood via centrifugation with repeat centrifugation and washing steps in buffered saline. The profile of the isotope bound within this cell population is presented in Figure 8 along with a proposed compartmental model and the average kinetic profiles from four subjects dosed intravenously with 100 nCi, 100 μg of a test compound.

The reservoir concept is affirmed in the concentration pattern for isolated PBMCs. Here, 3 of the 4 subjects displayed a drop to near zero drug concentrations at 12 hr, after which, ^{14}C levels rose again to display quantifiable drug concentrations out to the conclusion of sampling. The circulating mononuclear cells represent only 1% of the whole blood volume, yet the compound's uptake from circulating drug is easily quantified as the drug expanded into the large ($\geq 1000\text{ L}$) volume of distribution comprising cellular incorporation. Extra-vascularly labeled mononucleocytes were then seen entering the blood at 12 to 24 hours and remained quantifiable despite cellular equilibration.

These studies confirm that AMS is valuable quantifying the incorporation of labeled components during cellular growth or necrosis (folate) or for direct membrane transport and cellular retention (antiretroviral). PBMC analysis has been reported previously [60] and for measurement of intracellular phosphorylation rates (personal communication Dr. Graham Lappin). These concepts extend to the study of tissue specific uptake of any drug to a target tissue. For example, quantification of a labeled cancer drug within a tumor using a small-needle biopsy, penetration of an anti-infective into a dermal tissue, buccal scrapings or even bronchial mucosal lining after systemic delivery [61].

5. Dermal and Pulmonary Administration

Dermal and pulmonary routes of administration can benefit from AMS sensitivity and reduction in radiative exposures. The first clinical use of AMS explored the source of n-alkanes found in pathological dermal scale that were so ubiquitous as to raise questions of endogenous, perhaps associative, origin [62]. The small specimen capability of AMS allowed measurements of ^{14}C content on specimens from individuals and demonstrated that

the alkanes were actually localized metabolites of the petroleum-based jellies applied to relieve the irritations of dryness. In that instance, the lack of ^{14}C in the petroleum-derived preparations became the identifying “label”.

Dermal absorption of agricultural and industrial chemicals is a significant health issue for a large class of workers where the exposures are typically low but chronic over long periods, similar to the exposure routes of chronic therapeutics delivered by dermal patches, creams, or sprays. The human metabolism of ^{14}C labeled atrazine, the common crop herbicide assumed to have little effect on mammalian health, was the target of a high dose transdermal absorption and metabolism study designed for LSC quantitation. Low absorption and extensive metabolism prevented urinary profiling for metabolic biomarkers of the exposure by radiometric counting, but AMS successfully quantified radiolabel in the excreta (urine and feces) and identified major metabolites by co-chromatography after LC on-column injections of <4.5 pCi of ^{14}C extracts. AMS quantitation of total ^{14}C content in the bulk specimens correlated well with the original radiometric measurements after dilutions and AMS sensitivity was sufficient to quantify the changing suite of human urinary metabolites for 7 days post dose from single subjects [63-64]. These studies illustrate the potential for assessment of systemic exposure in transdermal studies using either course ADME readouts from excreta or more informative metabolite fingerprints.

The toxicology literature contains innumerable citations for aerosol inhalation and pulmonary instillation of ^{14}C labeled compounds in animals, but there are not good preclinical models for human inhalation of an aerosol. AMS sensitivity facilitates multiple sampling of parent and metabolite compounds over time in the same animal. A preclinical example investigated dogs after aerosol exposure to Moli1901, a 19 residue polycyclic peptide antibiotic [65]. A 45 nCi solution was delivered by intratracheal instillation (approximately 100 μg) into the left cranial lobe of the lung of beagle dogs resulted in retention of 64% of the dose in the left cranial lobe for up to 28 days as determined by tissue analysis after sacrifice. The concentration of Moli1901-related material in blood and plasma declined at first and then rose toward the end of the period, leading to the conclusion that the compound was being removed more slowly from blood than it was being added by absorption from the lung or from the gastrointestinal tract after swallowing. In this scenario the half-life for lung clearance would be approximately 40 days, highlighting the importance for radiological doses in any potential human to be as low as possible.

AMS-based inhalations studies promise to answer many development questions directly: 1) direct assessment of the rate and extent of drug removal from the lung as inferred from the plasma concentration and excretion profile; 2) kinetic assessment of systemic absorbed dose, since pulmonary drug doses are likely to be cleared/excreted much more slowly than drug which is swallowed; and 3) the direct measure of the metabolite profile of the pulmonary exposed drug. In one of the few studies known to have a pulmonary “target”, Lappin and coworkers quantified the appearance of ^{14}C -microdose of AR-709 into key lung compartments, epithelial lining fluid, alveolar macrophages and bronchial mucosal lining following intravenous administration of a 200 nCi dose of the anti-microbial (personal communication). While not a ^{14}C inhalation study per se, the study highlights the remarkable ability of AMS to quantify labeled drug in tissues and fluids that would not normally be accessible to ^{14}C -radiolabeled study using less sensitive detection methods.

A principal obstacle to human inhalation studies will lie with manufacturing: The manufacture of aerosols is technologically sophisticated and the introduction of ^{14}C labeled drug presents practical difficulties that will have to be addressed on a case-by-case basis. Lowering the compound’s specific activity and total radioactivity can reduce, although not remove, the precautions necessary for radioactive manufacture of aerosolized mixtures.

6. Exploring Pediatric Drugs

Up to 80% of medications used in children are without adequate dosing or efficacy information, also called off label use, due in large part to the lack of sensitive analytical methods appropriate for the special demands pertinent to pediatric studies [66-67]. AMS sensitivity enables kinetic and metabolic surveys of such special populations who must be shielded from high chemical and/or isotopic doses using typical sample sizes of 20 μ l of plasma/serum or 100 μ l of urine. These specimen amounts are available through indwelling catheters or heel sticks, and spot urine collections. Chemical doses may be only nanograms of compound, even at low specific ^{14}C labeling while radioactive exposure from the dose is again equal to only a low factor of the child's natural ^{14}C content, adding no more risk from radiation exposure than another day of life already provides. Three known application highlight these advantages for exploratory research in pediatric pharmacology.

^{14}C -urea breath test is a non-invasive, common technique to detect gastric *Helicobacter pylori* infection which was first available to children (7 to 14 years old) through AMS quantitation [68]. In 2002, the study was repeated in younger children from 3-6 years old, who were administered 12 nCi (440 Bq) of ^{14}C urea, a dose 250 times lower than the amount normally administered to adults [68]. The study showed that nano-dose ^{14}C -urea was sufficient to quantify gastric infection enabling repeated screening investigations with ^{14}C -urea in whole families, including children [69].

A second example arises from international nutrition. Vitamin metabolism in children is an important international health issue, with vitamin A supplementation having significant impact on childhood mortality [70]. Absorption, retention, and urinary excretion of ^{14}C -retinol (as an ester) was investigated by AMS in preschool Zambian children selected because they were unresponsive to regular vitamin A supplementation programs. Enrolled pre-school Zambian children were provided a 20 nanoCurie tracer of ^{14}C -retinol by mouth in conjunction with either 60 mg or 5 supplementation doses to assess absorption and retention in conditions that mimic high dose fortification and normal intake levels. Excreta were analyzed for total ^{14}C by AMS at a U.S.-based facility. Statistically insignificant differences in the urinary and fecal excretion patterns led to the conclusion that the vitamin A was adequately absorbed and normally retained in both groups, although absorption showed an inverse correlation to recent fever. Malabsorption would not explain the apparent low circulating vitamin A values in this sub-population, prompting studies focused on metabolic differences rather than simple absorption.

Loma Linda University Children's Hospital and coworkers recently reported preliminary results of an on-going study in preterm neonates (birth to 1 mo. in age) with the compound Ursodiol (Actigal®), used in the treatment of cholestasis. Pharmacokinetic profiles and dose proportionality were investigated in infants admitted to the Neonatal Intensive Care Unit (NICU) using three dosing levels with 48 hr interval between successive doses [71]. Plasma kinetic profiles of the ^{14}C concentration after ^{14}C -ursodiol dosed gastrically at the three dose levels (1, 3.3, and 10 nCi corresponding to 7.8, 25.7 and 78 ng, respectively) in three pre-term infants is shown in Figure 9. Wide physiologic variability was expected and observed in this small cohort of a population which had not yet reached post-natal homeostasis. The 37 Bq (1 nCi) of ^{14}C was sufficient to follow this nano-dose above LLOQ for at least 4 days in even the lowest absorbing subject. The LLOQ (six times the standard deviation of the predose blood draws) corresponds to 0.4 pM of the ^{14}C -ursodiol. The enterohepatic recycling of this natural bile acid is distinctly recognizable in two of the subjects by the return of signal at 24 hours.

This population is particularly sensitive to blood draw volumes. However, for an infant at the minimum weight requirement for this study (1.5 kg), the amount of blood drawn (< 600 μ L in 8-12 single-drop draws) was equivalent to approximately 3.4% of the infant's total blood volume, safely below the recommended ceiling value of 5% (within 24 hr period).

7. Drug-drug and Drug-Environment Interactions: Co-Administered Compounds

Drug-drug or drug-environment interactions (DDI, DEI) are addressed late in drug development, after a compound's biochemistry is determined in controlled clinical trials. Interactions among administered compounds are difficult to quantify even at those larger therapeutic doses, but the quantitative sensitivity and precision of AMS enables protocols that reveal modifications of pharmacokinetic and pharmacodynamic behavior for labeled compounds at low exploratory doses of both the compound and co-administered interacting compounds. Figure 10 shows quantitation of 1 μ g/kg (p.o.) 14 C-diisopropyl-fluorophosphate (DFP) covalently bound in murine tissues with no co-exposures and after prior sub-chronic administration once per day for 5 days of the pesticides parathion (PTN) and permethrin (PER) at 1 μ g/kg (p.o.) [72]. The pesticide exposures significantly increased the amount of DFP, a miotic pharmaceutical often used as a research analog of organophosphate pesticides and nerve agents, bound to the cholinesterase in the brain, suggesting a possible link between cholinergic pesticide exposure and increased susceptibility to very low amounts of nerve agents among certain troops after destruction of nerve gas depots [73-74]. The study further demonstrated that daily doses of the protective transient cholinesterase inhibitor, pyridostigmine bromide, sub-chronically dosed at 50 μ g/kg prior to 14 C-DFP administration, produced a significant 15% decrease in brain-bound DFP. Since pyridostigmine does not cross the blood brain barrier without insult [75], this decrease is attributed to increased enteric velocity of the orally dosed DFP induced by the pyridostigmine parasympathomimetic that decreased absorbed DFP dose, rather than to direct competition for cholinesterase binding. Concentrations of DFP, pyridostigmine, and the pesticides were too low in this work to have achieved competitive binding concentrations on any of the large complement of body esterases. These very low dose levels were capable of measurable physiochemical interactions between dosed compounds that could not have been reproduced by *in silico* or *in vitro* methods since the interaction was physiological rather than biochemical.

Covalent binding to DNA, creating potential mutagenic adducts, has long been a primary focus of quantitation by AMS, since these adduction events are rare (10^{-12} - 10^{-8} adducts/base), the amount of DNA available from clinical samples is small, and no other technologies offer the needed quantitative sensitivity. These quantified adducts are then used as probes of environmental or chemical exposures that modulate potential mutagenicity. Dingley, et al. [76] showed that the phytochemicals chlorophyllin, and phenylethylisothiocyanate (PEITC) were effective at reducing DNA adducts in one or more rat organs from the common food-borne carcinogen, phenyl-imidazo-pyridine, while genistein, and lycopene actually increased adduct levels in some organs. Changes in circulating concentrations of the carcinogen were also noted, indicating physiochemical responses independent of specific biochemical interactions with macromolecules. They further related these responses to up and down regulation of certain enzymatic activities providing mechanistic hypotheses for the observed physiochemical modulations.

Acrylamide is tumorigenic in rats [77], but not in mice [78] while constituting a neurotoxin in humans without apparent carcinogenic potential [79]. Its presence in the human diet as a consequence of high temperature interaction of amino acids with reducing sugars, especially in popular deep-fried foods, makes it a concern for human health. Xie and coworkers used

AMS to quantify significant modulation of covalent acrylamide attachment to liver DNA as well as blood (hemoglobin) and reproductive (protamine) proteins [80] by phytonutrients extracted from garlic (diallyl trisulfide), tea (polyphenols), and Japanese knotweed or wine (resveratrol), as well as the modified amino acid N-acetyl-cysteine in mice after human-exposure-equivalent doses. Figure 11 shows that such dietary components can reduce macromolecular binding of acrylamide by as much as 40%. The authors consider both DNA and protein macromolecules since DNA adducts arise from the glycidamide metabolite while proteins are attacked by the parent acrylamide. A metabolic inhibition that modifies this balance could be recognized by such DNA/protein comparisons. Both distributed creatine kinase and glutathione S-transferase were seen to be protected from acrylamide-induced depletion by phytonutrients, especially tea and garlic extracts, providing mechanistic guidance for the physiochemical response to the interactions between the toxin and the tested substances. The sensitivity and specificity of ^{14}C quantitation by AMS can be applied to various probes of DDI and DEI as long as the interesting quantity can be reflected by retained ^{14}C content in the living animal, including humans.

8. Absolute Bioavailability

The simultaneous dosing of an intravenous microdose of the labeled drug with a clinical dose administered by an alternate route (e.g. oral, dermal, sublingual) to determine the human clearance, volume of distribution and absolute bioavailability is a more recent and expanding application of clinical AMS measurement [81]. The *in vivo* mixing of unlabeled and labeled material is also referred as microtracing since total exposures are at therapeutic levels. This approach has broad utility and is seen by some as underutilized [29]. Intravenous pharmacokinetics along with those from the oral route for solid and liquid dosage forms allows determination, with modeling and deconvolution, the *in vivo* absorption and dissolution rate, establishing the *in vivo* platform for the *in vitro/in vivo* correlation. An excellent presentation of the underlying pharmacokinetic principles and applications were provided by Lappin and Stevens [31]. In the approach, the dose normalized Area-Under-the-Concentration-Time-Curve $\text{AUC}_{0-\infty}$ for an intravenous ^{14}C microdose is directly compared to the $\text{AUC}_{0-\infty}$ of the unlabeled active drug delivered by the intended route (see Figure 12). The respective areas provide estimates of absolute bioavailability (F) as well as other key parameters. This ^{14}C -microdose design brings at least five advantages to this study over stable isotope or two-way cross over designs.

1. Non-perturbing: The microdose is too small to perturb the kinetics of the extravascular dose and formulation is simplified.
2. Dosimetry: Intravenous enabling animal studies (tissue dosimetry, local tolerance) are not needed.
3. Relevant kinetics: The plasma compartment mixing of the micro and macro doses ensure equal Clearance for the two doses.
4. Metabolism: The ^{14}C - microdose enables determination of clearance, routes of excretion and second-pass metabolism.
5. Cost efficient with statistical power: The study delivers in a single arm what used to require two tests with a cross-over design and in between wash-out periods. Individuals serve as their own control, improving statistical power.

An early demonstration of a single exposure absolute bioavailability study was performed by Sarapa and coworkers on Nelfinavir, an antiretroviral compound, in healthy volunteers [82]. The study sought an explanation for the moderate oral bioavailability of the drug with the intent of determining an improvement could be addressed through formulation development. The results of two tests on the first and eleventh days of chronic unlabelled

exposures in 6 subjects determined that the decrease in Nelfinavir with daily dosing (from 0.88 (day 1) to 0.47 (day 11)) was attributable to metabolic enzyme induction. One major metabolite, AG1402, was observed only after oral dosing and did not carry the radiolabel, suggesting that it was likely only produced in the gastrointestinal tract. The moderate bioavailability due to first pass metabolism would suggest that an improvement in formulation would have little benefit.

Similar study designs enable reinvestigations of approved drugs for drug-drug interactions in the context of on-going therapy, and for optimization of individual choices in compounds or dosing schedules. Such quantitative metabolic information can equally be extended to assessing the relative role of the pharmacogenomic and environmental factors in the absorption and disposition of a wide variety of agent after either single doses or under steady-state conditions as shown for Nelfinavir. Emerging data from the European Microdosing AMS Partnership Programme or EUMAPP has several examples of absolute bioavailability as applied to various marketed compounds [40]. Lappin and coworkers [39] recently released the results of a triple cross-over study for the molecule fexofenadine, a molecule chosen to represent an unmetabolized transport substrate (P-gP and OATP). The absolute oral bioavailability of fexofenadine was 0.35 (microdose 0.41, therapeutic dose 0.30). Despite a 1200-fold difference in dose of fexofenadine, the microdose predicted well the pharmacokinetic parameters following a therapeutic dose for this transporter dependent compound.

9. Diagnostics/Biomarkers

Several promising clinical applications of AMS for diagnostics recently appeared. We (Vitalea Science) have been developing an assay for vitamin B12 absorption in humans to replace the Shillings test which involved high levels of radioactivity and 24 hr urine collections [83]. Vitamin B12 is essential to prevent anemia and likely is involved in dementia. Absorption of the vitamin is abrogated in pernicious anemia, an autoimmune disease that destroys parietal cells in the stomach that secrete intrinsic factor, a crucial transporter for the normal absorption of B12. An oral microdose and the associated plasma response using a single drop of whole blood will inform the clinician if absorption is normal or compromised. The test molecule was not amenable to chemical synthesis, but was easily produced via bioexpression using a modified bacteria (Figure 13). This “simple” system provides material for hundreds of human tests using bench scale synthesis due to the low radioactivity requirement for the AMS assay.

Cancer chemoresistance to a therapeutic greatly increases complexity and cost of cancer patient management. AMS has long quantified DNA binding to DNA, one of its first biomedical applications [5] and small doses (microdoses) of ^{14}C -labeled cancer drugs often exert their effect by similar covalent DNA modification (Figure 14). The extent of binding to DNA isolated from a biopsy sample at some period after a microdose may be predictive or prognostic of the proper course of treatment. One class of chemotherapeutics is the platinum-based drugs, such as cisplatin, carboplatin and oxaliplatin. Resistance to these drugs is common and poorly understood, but evidence suggests that it is influenced by variation in cellular rates of drug influx, export and the formation and repair of platinum-DNA adducts. Response rates in humans are as low as 25-30% for non small cell lung cancer, and 50% for bladder cancer. Many patients receiving platinum-based therapies often derive no benefit and experience a loss of critical time in commencing with a more appropriate treatment. Sensitive assays are under development using in vitro cell models, and human microdosing studies are planned in the near future [84] [84]. The assays currently developed have the sensitivity to detect 1 attomole of Pt-DNA adducts per μg of DNA, provided the drug has a radiocarbon label [85-86]; these diagnostic specimens are

harvested from left over tissue biopsy samples that are currently required to be obtained in standard clinical oncology practice. The drug-DNA damage could be compared to other molecular correlative data, such as RT-PCR of specific DNA repair genes, in order to provide maximal predictive capability.

There is substantial precedence for this concept from the early and on-going genotoxicity work. Indeed, AMS sensitivity was first applied in biochemistry to the comparative quantitation of reactive metabolites, metabolites that are such strong electrophiles that they have undetectable concentrations in circulation. Brown et al. provide an overview of these efforts in 2005 [87]. The ability of these direct measures of rare binding events is expected to serve the biomedical community through the development of sensitive diagnostic tests as described for the platinum drugs discussed above.

10. Evolving Technology - Instrument and Sample Introduction Methods

Early AMS instruments originated as very large complex instruments which required well staffed scientific laboratories to operate them. However, AMS instruments specifically designed for the measurement of carbon isotopes were developed that reduced the energy required for destruction of molecular isobars, reducing size and complexity. These instruments reduced the cost and overhead involved in AMS ^{14}C concentration measurements but they still had operational/safety issues with the exposed high voltages. Multiple ion scattering now eliminates molecular isobars at voltages of 200 kV[88-90]. The reduction in accelerator voltage allows the collision cell to be insulated by vacuum rather than insulating gases, and the voltage supplied by an off-the-shelf power supply. These advances allowed the AMS to be much smaller ($2.5 \times 3 \text{ m}^2$), easier to maintain, and have improved safety profile (Figure 15).

There is reluctance on the part of some groups to become directly involved with AMS technology because the production of graphite is seen as difficult despite a robust *in situ* production method described by Ognibene and coworkers [91]. This is a valid barrier to entry as the materials and other required equipment are not “off the shelf”. In most circumstances, sample preparation experience, either through internship or the use of consultants, will have to be acquired to set up an AMS facility and generate high quality graphite. One option to simplify sample preparation is the direct feed of gaseous CO_2 . This approach of direct feed of CO_2 to a negative ion source was demonstrated in 1984 [92]; subsequent attempts to use this gas in carbon dating [93] and in biochemistry [94-95] have suffered from reduced spectrometer throughput or decreased precision, to the point that CO_2 has seldom been used in carbon spectrometers except for particular systems designed for molecule-specific GC-AMS [96]. Active research in this area is underway at several groups and the picture may change in coming years as improvements in the on-line combustion of a samples to CO_2 improve. Until that point, the bulk of AMS measurements will likely continue to reside with highly specialized contract research organizations, universities, or national labs.

Other emerging technologies also hold promise for carbon isotope concentration measurements such as the Laser Assisted Ratio Analyzer (LARA) system [97]. The LARA system uses laser resonances in CO_2 to analyze carbon isotope ratios and promises to be a bench top sized system capable of being operated by a single technician. LARA is still in development and has many hurdles to overcome, but it may someday compliment or replace AMS technology. However, AMS instruments will continue to dominate high sensitivity radioisotope concentration measurements for many years to come.

11. Concluding remarks

Low dose AMS analyses contribute to key objectives in pharmaceutical research. Human ADME can be established at microdose levels and the results can be used in selection of candidates, or for pro-drug or formulation optimization using iterative designs. The utilities of isotopically labeled compounds are also brought to bear on topical, pulmonary, dermal or other low absorption routes of administration previously excluded by potential radiological concerns. Sensitive populations such as children, commonly excluded from routine study, can be studied where the risk is limited only to the blood drawing and not radiological exposure. The use of an intravenous tracer to show absolute bioavailability for oral or other routes of administration or to quantify tissue targeting drug delivery vehicles is a unique and useful application of AMS sensitivity.

Microdosing is currently underutilized out of fears over non-linearities in pharmacokinetics. This is unfortunate as knowledge of low dose physiochemistry, metabolism and disposition would certainly lead to better candidate selection and allow early go/no go decision making. The rationale is simple: human data are inherently more relevant than animal data in the selection of compounds developed for human use. To quote Robert Ings, an early adopter of microdosing: "Microdosing is only one tool in the drug developer's too box, but when used appropriately and in the context of all the other tools available, it will have an increasing impact on the timelines and quality of drug development" [29].

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Figure 1.

Schematic of the principles a triple collector Accelerator Mass Spectrometer. Core concepts of ^{14}C -AMS are : 1. Negative ion production to eliminate the atomic isobar; 3. High energy collisions to eliminate molecular isobars; 5. Ion identification of single atoms for specificity of response; Normalizing to stable carbon isotopes with equivalent ion pathways eliminates quantitative effects from variable ion emission and transmission.

Figure 2.

Pathways of β -carotene absorption and metabolism. Enzymatic cleavage in the intestinal cells produces retinyl esters (vitamin A) which are transported by the lymphatic system into circulation. Absorbed β -carotene and retinyl esters are cleared by the liver and other tissues from circulation before being resecreted into blood either intact or as retinol. The metabolism of this lipophilic "provitamin A" compound is complex and variable across species.

Figure 3.

Absorption and distribution kinetics for carotene, retinyl esters and retinol following a high fat dosing meal. A 5.5 hr lag in the first appearance of ^{14}C was observed perhaps due to delayed gastric emptying. Similar concentration vs. time plots for retinyl esters and β -carotene reveal a shared lipid transporter pathway. Retinol takes an entirely different course as it is the product of the liver secretion of absorbed retinyl esters packaged with retinol binding protein.

Figure 4. Loss of ^{14}C in feces and urine following a small (1 nmol) oral dose of ^{14}C - β -carotene in an oil-based banana cocktail to an adult woman before and after vitamin A supplementation. Vitamin A supplementation (after plots) markedly improved absorption (decreased fecal loss) and reduced urinary elimination.

Figure 6. Semi-log plot of plasma warfarin concentrations (geometric mean, SE) versus time for 100- μ g (open squares) and 5-mg (solid squares) oral doses normalized to 1-mg dose. Warfarin is a synthetic derivative of coumarin, a chemical found naturally in many plants (with permissions). A lack of concordance between the PK for the low and therapeutic doses was observed. Low dose kinetic behavior is being explained using physiologic-based compartmental models (PBPK models).

Figure 7.

¹⁴C content of plasma and red blood cells (RBC) of human volunteers (n=13) from 5 minutes to 200 days after a single 3.7 kBq (100 nCi) dose of ¹⁴C-folic acid. RBC incorporated labeled folate during the ≈24 hours of high plasma content and revealed a 6.1 day maturation for RBC. RBC lifespan is clearly 120 days; with permissions [14].

Figure 8.

Proposed graphical model to explain kinetics of a microdosed HIV that is analyzed in harvested white blood cells (PBMCs) from whole blood. The unusual cellular kinetic behavior of the “drug-labeled” cells is attributed to the expansion of the cells into compartments outside the accessible plasma that return approximately 12 hr post dose. Some of the drug return may also be from extracellular fluid spaces.

Figure 9. Plasma kinetics of 3 consecutive doses (1 nCi, 3.3 nCi, 10 nCi) of ^{14}C -ursodiol in neonates. Results from AMS analyses show within subject dose proportionality and significant difference in plasma re-uptake and elimination between 2 subjects).

Figure 10.

A.) Subchronic 5 day exposures of mice to pesticides parathion and permethrin, singly and together, at 1 $\mu\text{g/kg}$ significantly (*) increased covalently bound ^{14}C -DFP levels in brain tissue after a 1 $\mu\text{g/kg}$ single exposure. B.) A reversible esterase inhibitor, pyridostigmine bromide (PYB), subchronically dosed at 50 $\mu\text{g/kg}$ significantly decreased this binding by 15-20% through a parasymphomimetic physiologic mechanism, since all compounds combined were insufficient to cause competitive inhibition, particularly in brain which is inaccessible to PYB; with permissions [72].

Figure 11.

Quantitative decreases in binding of ^{14}C -acrylamide to DNA were measured in mouse tissues after exposures to plant extracts, including diallyl trisulfide (garlic), resveratrol (Japanese knotweed, red wines), and tea polyphenols. Asterisks indicate significant decreases ($p < 0.05$) with the numbers indicating the percentage decrease in adducted ^{14}C ; with permissions [78].

Figure 12.

Procedure for the determination of absolute bioavailability where a ^{14}C -labeled intravenous dose is infused shortly after the extra-vascular therapeutic dose is administered. As the ^{14}C “microdose” mixes with recently absorbed clinical dose, relevant kinetics are ensured; with permissions [31].

Figure 13. Biochemical pathways of *S. enterica* serovar Typhimurium pathways that conjugates two essential exogenous precursors to produce [¹⁴C]vitamin B₁₂: [2-¹⁴C]-dimethylbenzimidazole and cobinamide dicyanide.

Figure 14.

Simplified scheme of the reaction with oxaliplatin with DNA. AMS offers the dynamic range and quantitative capacity to measure both high therapeutic concentrations of the labeled drug entering and exiting the cells and the low concentrations of DNA adducts prior to and following DNA repair; with permissions [99].

Figure 15.

The compact and vacuum insulated BioMICADAS AMS spectrometer. The picture shows a 200 kV accelerator (center) between ion source and initial mass spectrometer (right) and a magnetic-electric mass spectrometer before the ion counter (left). This instrument was developed at the Swiss Federal Institute of Technology for biomedical applications [88].