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# Analytical Validation of Accelerator Mass Spectrometry for Pharmaceutical Development: the Measurement of Carbon-14 Isotope Ratio.

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February 8, 2010

Bioanalysis

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1 **Analytical Validation of Accelerator Mass Spectrometry for Pharmaceutical**  
2 **Development: the Measurement of Carbon-14 Isotope Ratio.**

3

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5

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9

10 **Keywords: Accelerator mass spectrometry (AMS), Liquid Chromatography with AMS (LC-AMS), AMS Units**  
11 **of Measure, Isomolar Fraction and Equivalents.**

12

13 **Future Perspective: Accelerator** mass spectrometry (AMS) is an isotope based measurement technology that utilizes  
14 carbon-14 labeled compounds in the pharmaceutical development process to measure compounds at very low  
15 concentrations, empowers microdosing as an investigational tool, and extends the utility of <sup>14</sup>C labeled compounds to  
16 dramatically lower levels. It is a form of isotope ratio mass spectrometry that can provide either measurements of  
17 total compound equivalents or, when coupled to separation technology such as chromatography, quantitation of  
18 specific compounds. The properties of AMS as a measurement technique are investigated here, and the parameters of  
19 method validation are shown. AMS, independent of any separation technique to which it may be coupled, is shown to  
20 be accurate, linear, precise, and robust. As the sensitivity and universality of AMS is constantly being explored and  
21 expanded, this work underpins many areas of pharmaceutical development including drug metabolism as well as  
22 absorption, distribution and excretion of pharmaceutical compounds as a fundamental step in drug development.

23

24 **Abstract:** The validation parameters for pharmaceutical analyses were examined for the accelerator mass  
25 spectrometry measurement of <sup>14</sup>C/C ratio, independent of chemical separation procedures. The isotope ratio  
26 measurement was specific (owing to the <sup>14</sup>C label), stable across samples storage conditions for at least one  
27 year, linear over 4 orders of magnitude with an analytical range from one tenth Modern to at least 2000

28 Modern (instrument specific). Further, accuracy was excellent between 1 and 3 percent while precision  
29 expressed as coefficient of variation is between 1 and 6% determined primarily by radiocarbon content and  
30 the time spent analyzing a sample. Sensitivity, expressed as LOD and LLOQ was 1 and 10 attomoles of  
31 carbon-14 (which can be expressed as compound equivalents) and for a typical small molecule labeled at  
32 10% incorporated with  $^{14}\text{C}$  corresponds to 30 fg equivalents. AMS provides an sensitive, accurate and  
33 precise method of measuring drug compounds in biological matrices.

34

## 35 **Executive Summary**

36 **AMS provides accurate (93 – 103% accuracy observed over many sample types, range in isotope**  
37 **ratio, individual experiments and several years of observation), precise (observed imprecisions are 2 –**  
38 **5%), reliable measurements of  $^{14}\text{C}/\text{C}$  atom ratio that are an essential part of making AMS based**  
39 **measurements of labeled compounds concentrations in biological matrices as equivalents or as**  
40 **isolated compounds.**

41 **AMS correlates extremely well with conventional measure of  $^{14}\text{C}$ , provided both the comparative**  
42 **technique of liquid scintillation counting is accurately done and the AMS instrument is robustly**  
43 **designed across a wide analytical range, allowing direct comparison of results.**

44 **AMS is very stable across time and the background radiocarbon levels in coincident samples are**  
45 **highly uniform (less than 3% variation in coincident sample groups).**

46 **When used with appropriate, controlled chemical separations such as UPLC or HPLC, AMS provides**  
47 **the basis for very sensitive (fg-eq/mL), precise quantitations of individual compounds, whether as an**  
48 **identified analyte or as unknown, but quantifiable, metabolites.**

49

## 50 **1. INTRODUCTION**

51

52 Method validation proves that an analytical method is acceptable for its intended purpose. In recent years, conferences  
53 supported by the American Association of Pharmaceutical Scientists and the FDA refined the concepts behind  
54 providing validated bioanalytical data from chromatographic and ligand-binding assays in support of new drug  
55 development (1), but these assays offer only comparative quantitation requiring internal standards and/or calibration

56 curves. Accelerator Mass Spectrometry (AMS) is a method of directly quantifying the concentration of a rare isotope  
57 (< parts per billion, ppb) versus a common isotope in a uniform matrix derived from a defined biological sample.  
58 AMS shares many characteristics with quantitation by isotope decay counting (e.g. liquid scintillation counting, LSC)  
59 which also does not use internal standards or compound-specific calibration; but AMS offers much greater sensitivity,  
60 specificity, and versatility. Validation of AMS for pharmaceutical development adheres to the goals of the recent  
61 bioanalytical validation conferences but must rely on more analytically suitable guidelines from the United States  
62 Pharmacopeia (2), International Conference on Harmonization (3), and the Food and Drug Administration (4), (5) for  
63 a structure to perform and report such validations. Validation of any analytical method derives from trustworthy data  
64 on specificity, linearity, accuracy, precision, range, detection limit, quantitation limit, and robustness (6). This paper  
65 shows that AMS quantitation of an isotopic tracer or molecular label is a fundamentally validated measurement in its  
66 own right and that it can be used with bioanalytical separation instruments to provide valid bioanalytical quantitation  
67 for biomedical research and/or pharmaceutical development.

68

69 AMS developed over the past 3 decades for quantifying radioisotope concentrations in natural samples for specific  
70 isotopes whose halflives are so long that decay counting is very inefficient (generally, isotopes with halflives greater  
71 than about 100 years). Kutschera (7) provides an overview of the breadth of current AMS applications. AMS is most  
72 often applied to  $^{14}\text{C}$  for carbon dating archaeological or earth science samples. The progress in these areas is found in  
73 the proceedings of both international radiocarbon conferences and international AMS conferences held triennially and  
74 published in the journals: Radiocarbon (University of Arizona) and Nuclear Instruments and Methods B (Elsevier),  
75 respectively. Multiple interlaboratory comparison programs carried out by international radiocarbon dating facilities  
76 show that AMS is more accurate, precise, and robust than decay counting techniques, including LSC and gas  
77 proportional counting (GPC) (8). These programs covered  $^{14}\text{C}$  concentrations relevant to dating purposes: the part per  
78 trillion (ppt) to part per quadrillion (ppq) range of  $^{14}\text{C}/\text{C}$  concentrations that correspond to present day levels of  $^{14}\text{C}$  in  
79 living organisms back to organic residues that have been dead for fifty thousand years. AMS was driven by the desire  
80 for radiocarbon dating milligram sized samples whose natural decay rates were only 0.001 – 0.2 decays per minute  
81 (dpm). Reliable absolute quantitation of a single sample was emphasized, because these samples were small, unique,  
82 irreplaceable materials linked to a specific archaeological, social, or geological event, such as the medieval origin of a

83 linen cloth on which appears an iconic image (9). When biomolecular tracing with AMS was first demonstrated (10),  
84 procedures were developed from existing AMS processes for quantifying biochemical isolates for dating purposes  
85 (11) using the sample preparation methods and the spectrometer operations that were already suitable for geoscience  
86 chronometry.

87

88 A basic overview of AMS operation in biomedical measurements can be found in short form in (12) or in quantitative  
89 detail in (13). The value of AMS for drug development is generally discussed by Turteltaub and Vogel (14), Lappin  
90 and Garner (15), and Wilding and Bell (16). AMS counts specific isotopes using tandem isotope ratio mass  
91 spectrometry, but AMS is best thought of as an atom counter rather than a mass spectrometer. The typical concerns of  
92 molecular mass spectrometry, such as species ionization or matrix effects, are not present in the AMS method.  
93 Instead, the mass of the single analyte is well known (14 amu for  $^{14}\text{C}$ ) and all spectrometer parameters are stably  
94 maximized for the isotope(s) being counted. AMS measurements (for both earth and biological sciences) require the  
95 contamination-free definition of the assayed material and an isotopically non-fractionating conversion of the selected  
96 isolate to a uniform, matrix-independent form that readily makes negative elemental ions. These ions are transported,  
97 selected, and identified by stable spectrometric sectors that propagate the ions from a sample to both the rare and  
98 common isotope detectors without differential losses. Even the “mass resolution” of the several dipole magnets in an  
99 AMS are not important to valid operation of AMS. The comparison of a sampled isotope ratio to that of a National  
100 Institute of Science and Technology (US-NIST) traceable external reference standard quantifies an absolute isotope  
101 concentration for each measurement. AMS quantifies a  $^{14}\text{C}/\text{C}$  ratio which can be expressed succinctly by an  
102 internationally defined unit, “Modern”, which is approximately the concentration of natural  $^{14}\text{C}$  that is found  
103 throughout the atmosphere and living organisms (17). This  $^{14}\text{C}/\text{C}$  concentration (1.118 part per trillion) is expressible  
104 in many different units, independent the molecular weight or specific activity of a labeled compound under study.

105

106 We discuss the validation of  $^{14}\text{C}$  AMS analysis of biological materials for specificity, stability, linearity, range,  
107 repeatability, precision, accuracy, sensitivity, additivity, and robustness. We do not discuss here any chemical  
108 isolation procedures that may necessary as a complement to the AMS measurement to provide chemical specificity,  
109 most importantly extraction and chromatography.

110

## 111 2. VALIDATION MEASUREMENTS

112

### 113 2.1 Specificity

114

115 Specificity is the unequivocal detection of the compound under study in relevant samples independent of effects of  
116 impurities, matrices, or degradation processes. Specificity is most often achieved through molecular isolation or  
117 identification, often as separation in a chromatograph, mass spectrometer, or binding assay. AMS quantifies only a  
118  $^{14}\text{C}$  molecular label that is non-specific for the biochemical identity containing the  $^{14}\text{C}$ . Molecular specificity thus  
119 depends on sample isolation processes that limit the quantified  $^{14}\text{C}$  to those that are related to the studied compound.  
120 Unlike decay counting, AMS is specific to the isotopic identity (atomic mass and charge) of the detected atom, not its  
121 decay product. Other radioactive elements or optical noise (chemiluminescence, etc.) do not introduce sample-  
122 dependent non-specificity to the quantitation. Matrix interferences are completely removed from AMS quantitation  
123 through the homogenization of all compounds and matrices to a common inorganic form ( $\text{CO}_2$ , carbon) prior to  
124 measurement (18). This homogeneity is an essential property of isotope ratio mass spectrometry, insuring that the  
125 isotope concentration measured for any fraction of the sample is equivalent to the average concentration of the entire  
126 sample.

127

128 Combining secondary ionization mass spectrometry (SIMS) with AMS provided spatial information on  $^{14}\text{C}$   
129 concentrations (19), but the ionization of carbon atoms from an organic matrix is so inefficient that decay-based  
130 radiography is more sensitive, if time consuming. AMS is directly linked to separation instruments (GC, LC) by  
131 Lieberman, et al., 2004 (20) and Choi, et al., 2005 (21), but the chemical specificity must still be validated with  
132 respect to the chromatographic parameters and is not a general property of the AMS quantitation *per se*. AMS non-  
133 specificity for the sources of  $^{14}\text{C}$  in a sample means that biochemical isolations define the measured isotope ratio;  
134 natural sources of  $^{14}\text{C}$  must be taken into account; and unrecognized sources of  $^{14}\text{C}$  (contamination) must be  
135 scrupulously avoided or understood (13). The effects of natural or purposely introduced  $^{14}\text{C}$  concentrations that  
136 contribute to quantitation and estimations of error are covered below.

137

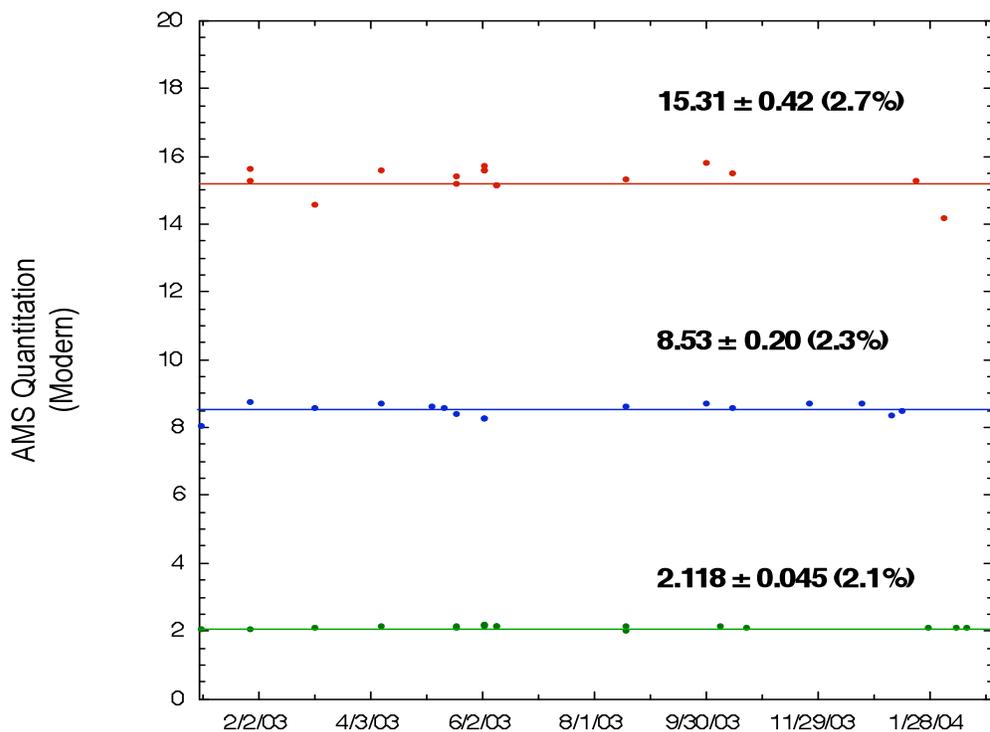
138 AMS specificity is thus best defined as an isotopic “instrument background” obtained by introducing a sample that  
139 should be comprised of fossil fuel derived carbon (no  $^{14}\text{C}$ ). Any counts identified as  $^{14}\text{C}$  in the detector may be faint  
140 levels of  $^{14}\text{C}$  memory in the ion source from previous samples or adsorbed atoms in the sample from the atmosphere,  
141 but most are stable carbon isotopes that fortuitously scatter at low probability ( $\leq 10^{-10}$ ) from the molecular breakup of  
142 background ions in the accelerator. AMS carbon dating extends to materials as old as 50,000 years before present  
143 (22), from which  $^{14}\text{C}$  concentrations of parts per quadrillion ( $1:10^{15}$ , ppq) are quantified. Biochemical AMS  
144 spectrometers are often operated at instrument backgrounds of order 10 parts per quadrillion, primarily including  
145 scattered non- $^{14}\text{C}$  ions. These scattered ions change little from sample to sample because all samples are reduced to a ,  
146 particular inorganic form, and this instrument background is subtracted as a component of the experimentally derived  
147 biological or chemical control samples. Thus, the specificity of the instrument for quantifying the isotopic label  
148 extends to the order of ppq.

149

## 150 2.2 Stability

151

152 Stability of an analytical method means that aliquots of the same sample provide equivalent quantitation after storage  
153 for various periods. The stability of AMS quantification over time arises from its lack of biochemical specificity,



154 since no chemical or physical degradation affects the isotope concentration in a stored aliquot unless a volatile  
155 component is generated and allowed to escape. Even the natural decay of the radioisotope is easily calculated and  
156 corrected but is not significant.  $^{14}\text{C}$  concentration decays at a rate of 1% every 83 years, and isotopic loss by decay is  
157 within any measurement errors for samples stored for decades. AMS stability relates only to the quantitation of the  
158 isotopic label within the total or a portion of a homogeneous suspension of the stored material. Chemical separations  
159 (e.g. metabolic profiles) of stored materials may diverge from initially measured profiles. This is not unique to AMS  
160 quantitation and is rightly a part of validation concerns for sample fractionation (chromatography, etc.) and storage  
161 profiles.

162

163

164 *Figure 1. Stability of AMS measurements of total  $^{14}\text{C}$  in three human serum samples is shown by periodic aliquots*  
165 *obtained from multiple freeze-thaw cycles throughout a year. CV's of the measured isotope concentrations were*  
166 *within 3%.*

167

168 Figure 1 shows the stability over one year of AMS quantitation of the isotopic label concentrations in three stored sera  
169 that underwent at least 15 measurements from frozen aliquots.. The coefficients of variation (CV) of the averages over  
170 that year were under 3% for all samples, and there is no significant trend versus time ( $r^2 = 0.067, 0.059, 0.0012$  top to  
171 bottom). The stability of AMS quantitation of the isotope concentration in homogenized fluids is enhanced by the  
172 independence of the measurement on the volume and amount of carbon in the measured aliquot. A uniform volume  
173 (20  $\mu\text{l}$ ) of the vortexed thawed samples were taken for each serum measurement in Figure 1, but any differences in the  
174 exact volumes have no effect on the isotope ratio measurement: a larger (smaller) volume would have proportionately  
175 more (less) of both the  $^{14}\text{C}$  and the total carbon of the original sample. The concentration of carbon per volume of  
176 serum is homeostatically constant in humans ( $\approx 42 \text{ mg/ml}$ ), and the specific activity of the labeled drug is presumably  
177 known, so that the quantified concentration of drug equivalents in the sera is just as stable as the measured isotope  
178 concentrations.

179

180 The stability of the fullerene filamentous powder (23), to which all samples at most AMS facilities are reduced prior  
181 to measurement, was tested on archived materials with the graphite's  $^{14}\text{C}$  concentration as  $0.7756 \pm 0.0041$  Modern  
182 in December 1988, as  $0.7850 \pm 0.0043$  Modern in 2006, a difference of 1.2%. The later measurement was higher than  
183 the initial one and may be due to contemporary atmospheric carbon adsorbing onto the fullerene powder over the 18  
184 years, although the sample was sealed in a screw-top gasketed vial and not previously opened. This particular form of  
185 graphite powder is especially absorbent of organic vapors, and care must be taken to store the prepared materials  
186 away from any sources of  $^{14}\text{C}$ -labeled volatiles (24). The difference of 1.2% is within a 2 SD uncertainty on the  
187 difference. This shows that samples can be prepared, reduced to elemental carbon, stored, and shipped for later AMS  
188 measurement without concern for chemical or isotopic changes over periods of years, let alone the more usual delay  
189 of days or weeks.

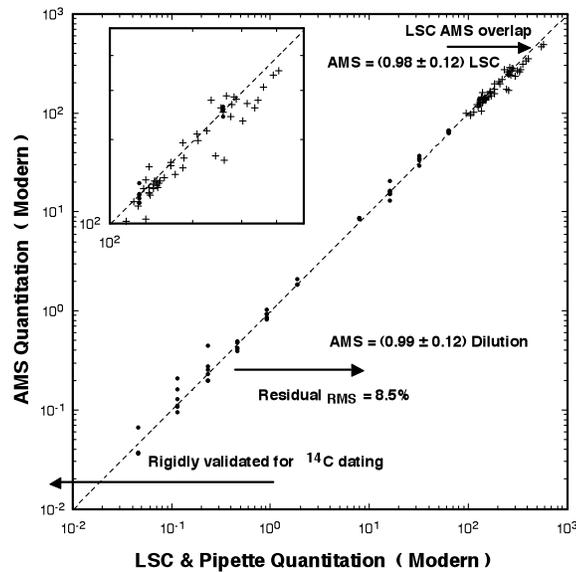
190

### 191 **2.3 Linearity and Range**

192

193 Linearity of an analytical method is a measure of the proportionality of the analyzed result to the amount of the  
194 analyte in the sample. AMS is designed to have absolute quantitation, and thus linear response, over a wide range of  
195 isotope concentrations. The linearity of the lower 3 orders of magnitude of the AMS range, ppq to ppt, are rigorously  
196 demonstrated by the regular International Radiocarbon Intercomparisons (8) and International Radiocarbon  
197 Calibrations (22) to assure carbon dating accuracy. Biomedical applications primarily (but not necessarily) quantify  
198 above the natural concentration of 1.2 ppt (Modern). Dilution series of biological fluids that begin at easily counted  
199 LSC concentrations are a common demonstration of linearity (25). We show such a plot in Figure 2 (solid dots) for a  
200 urine dilution series over a range of  $10^4$ . There is also a range of  $^{14}\text{C}$  concentrations above 100 Modern for which  
201 milliliter urine samples are easily quantified by LSC while smaller aliquots (100  $\mu\text{l}$ ) are quantifiable by AMS. Over  
202 50 human urines in this range were measured by both methods (shown as crosses in Figure 2 and the inset plot). The  
203 slope of these data matched those of the dilution series. The slopes and errors are consistent with a unity relationship.  
204 Certain difficulties in comparing LSC and AMS urinary  $^{14}\text{C}$  concentrations are discussed below.

205



206

207

208 *Figure 2. Linearity of AMS quantitation over 5 orders of magnitude is demonstrated by a urine dilution series (3 or 6*  
 209 *samples at each concentration), 50 direct comparisons between LSC and AMS measurements of the same materials,*  
 210 *and by the well documented range of <sup>14</sup>C dating down to ppq concentrations.*

211

212 Linear regressions over the large dynamic range available from AMS are not possible, because higher doses/responses  
 213 dominate the minimization of residuals in deriving the fits. The straight line in the log-log plot Figure 2 indicates that  
 214 the relation is a power law and the best fit is found as the mean and standard deviation of the ratio of the AMS  
 215 measure to the direct LSC value ( $0.98 \pm 0.12$ ) or the calculated dilution factor ( $0.99 \pm 0.12$ ), showing a direct one to  
 216 one proportionality. A linear regression of the logs of the data (forcing the regression program to minimize the  
 217 fractional residuals for all data points), confirms linearity (exponent of power law =  $0.989 \pm 0.011$ ,  $r^2=0.9965$ ) The  
 218 median of the residuals to a constant slope between AMS and LSC/Dilution in Figure 2 is 0.0% (sic) and the root  
 219 mean squared (RMS) residual of all the data is 16% (8.5% above 0.8 Modern), showing that AMS is as linear as LSC  
 220 over this range. Sub-Modern concentrations of <sup>14</sup>C are validated through radiocarbon dating intercomparisons using  
 221 LSC systems that are much more advanced than common systems in biochemical or environmental laboratories. The  
 222 data in Figure 2 mean that AMS has been directly compared to LSC without dilutions or enrichments over 6 orders of  
 223 magnitude, except from 1 Modern to 100 Modern. The urine dilution series spans this region with a reliable linear  
 224 regression, validating linear AMS quantitation.

225

226 FDA guidelines suggest testing linearity for a target concentration of analyte by quantifying a tight range of three  
227 standards, such as 80, 100, and 120% of target, or a broader range of 50%, 100%, and 150%. This may be useful in  
228 specific AMS measurements of biological materials that have undergone extensive, possibly non-quantitative,  
229 isolation or purification, but the design and history of AMS indicates that the absolute quantitation of  $^{14}\text{C}$  in the  
230 presented sample material is linear well within 20% at all times. Thus, AMS is a valuable tool for drug development  
231 during which response may not be well predicted, requiring multiple calibrations of other instruments. With wide  
232 range applicability, quantitation is assured even if quantified results fall well outside the range of expected  
233 concentrations.

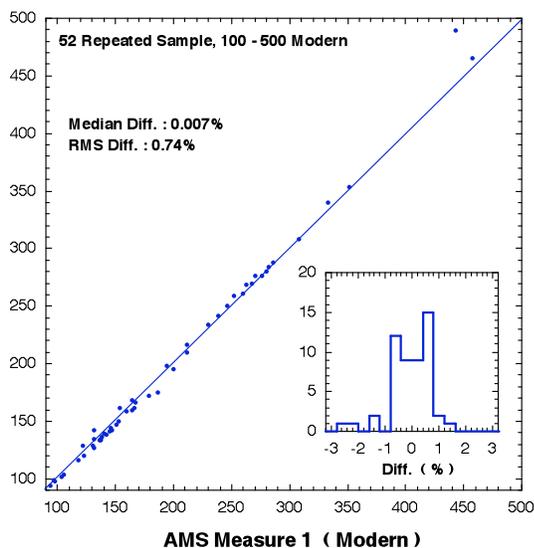
234

#### 235 **2.4 Repeatability, Precision, and Accuracy**

236

237 Precision and accuracy are individually addressed but are intimately entwined in a quantitative technology for which  
238 multiple calibration materials are not widespread. Precision is a measure of reproducibility from multiple measures of  
239 several samples, preferably spanning a period of time and a range of concentrations. Accuracy is assessed from  
240 multiple measures of reference materials with well known concentrations or from direct comparison to already  
241 accepted quantitative methods. Accuracy is implied once a method's specificity, linearity, repeatability, and precision  
242 are validated. First, the degree of repeatability of the instrument measurements was found to show that precision and  
243 accuracy can be reliably quantified by AMS.

244



245

246 *Figure 3. Fifty two urine samples having high  $^{14}\text{C}$  concentrations (90-450 Modern) were remeasured 10 days apart.*

247 *Reproducibility averaged  $<0.01\%$ , with an RMS difference of  $0.74\%$  between the two measurements. The inset shows*

248 *that the frequency distribution of residuals was not Gaussian.*

249

### 250 **2.4.1 Repeatability**

251 The inherent repeatability of AMS isotope ratio measurement is found by measuring a set of prepared and mounted

252 samples on different occasions, preferably separated by several days under normal operating conditions. Figure 3

253 shows repeat normalized measurements of carbon samples derived from human urine that were obtained 10 days

254 apart after the spectrometer had undergone several power cycles and measurement runs for other materials. The

255 samples were removed from the ion source and stored in an argon-filled plastic bag. Samples are bombarded by

256 cesium metal in the ion source and CsOH forms during removal in air that reacts with  $\text{CO}_2$  in the air, incorporating

257 atmospheric  $^{14}\text{C}$  into an exposed sample. For this reason, samples are seldom retained and remeasured after being

258 cesiated, but samples with high  $^{14}\text{C}$  concentration are minimally affected by absorbed atmospheric  $\text{CO}_2$ . Figure 3

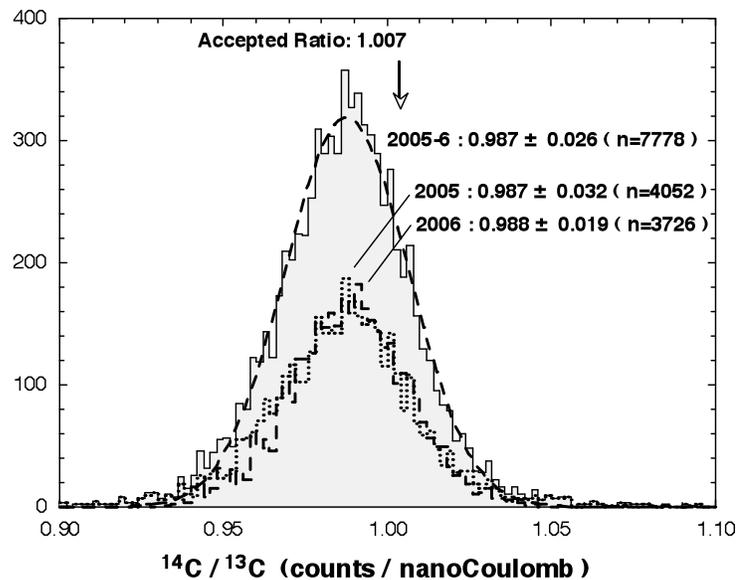
259 shows that normalized measurements of samples from 90 to 450 Modern are reproducibly measured to a median

260 difference of  $0.007\%$  with an RMS difference between the two sets of  $0.74\%$ , showing the high degree of

261 repeatability of the spectrometer and its operation.

262

263 The repeatability of sample combustion, reduction, and the routine operation of the spectrometer was demonstrated by  
 264 measurements of the external standard that was included in every set of samples (64) loaded into the spectrometer.  
 265 Figure 4 shows the frequency distribution of nearly 8000 isotope ratio measurements on approximately 2000 separate  
 266 samples of a commonly used external standard, IAEA C-6 sucrose (26). The theoretical ratio of  $^{14}\text{C}$  to  $^{13}\text{C}$  in this  
 267 material is  $1.61 \times 10^{-10}$  which is expressed as  $^{14}\text{C}$  per nanoCoulombs (nC) of accelerated  $^{13}\text{C}$  ion current through  
 268 division by the number of nanoCoulombs per singly charged  $^{13}\text{C}$  ion (the charge on the electron, a physical constant),  
 269  $1.60 \times 10^{-10}$  nC/ion, yielding an expected isotope ratio of 1.007 cts  $^{14}\text{C}$  per nC  $^{13}\text{C}$ . Figure 4 shows that these  
 270 measurements (made over the course of 2 years) have an average raw ratio of 0.987, 2.0% less than the theoretical  
 271 value as expected from the greater (but constant) scatter loss of the slower  $^{14}\text{C}$  through the spectrometer. The  
 272 distribution of values closely follows a Gaussian with a CV of 2.7%. These measurements were made under a variety  
 273 of operators, operating conditions, and graphite production sources throughout two years, demonstrating the inherent  
 274 repeatability of the AMS process from combustion to measurement over long periods despite operator biases or  
 275 spectrometer variances.  
 276

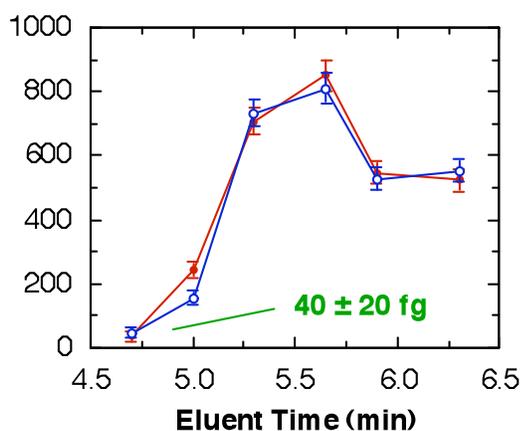


277  
 278 *Figure 4. The frequency distribution of the raw isotope ratio for 4,450 measurements of a  $^{14}\text{C}$  standard over the*  
 279 *period of two years shows a 2.7% standard deviation in a nearly normal distribution.*

282 AMS quantifies not only total isotopic label in defined samples of collected tissues or fluids, but also quantifies  
283 metabolic profiles and other small biochemical isolates. Eluents of LC separation, especially UPLC, contain very little  
284 carbon after solvent removal by centrifuge evacuation. An accurate aliquot of diluent carbon is added to the dried  
285 eluent to obtain enough mass for processing and for accurate quantitation of the tracer present in the fraction  
286 independent of variations of the fraction's natural carbon content (13). Figure 5 exemplifies the repeatability of this  
287 entire separation process followed by sample dilution, combustion, reduction, and measurement using separate UPLC  
288 runs performed on different days. The tracer level for each fraction is found by subtracting the known  $^{14}\text{C}$   
289 concentration of the diluent carbon from the measured concentration for each fraction. The tracer level is then  
290 converted to grams of equivalent drug using the isomolar fraction (fraction of molecules containing a  $^{14}\text{C}$ ) of the  
291 labeled compound and its molecular weight. The uncertainties in both the sample isotope ratio and the carrier isotope  
292 ratio are propagated to provide a quotable uncertainty in the difference, and hence in the specific metabolite quantity.  
293 Thus, AMS provides highly quantitative metabolite profiles with only external standards and no concern about  
294 differential ionization, the availability of internal standards, or compound-specific calibration curves. Note, especially,  
295 that zero drug equivalent (within stated error) is within the capability of AMS measurements, since the diluent carbon  
296 does have a known level of  $^{14}\text{C}$ . The introduction of a quantitative guidance for toxicity testing of circulating human  
297 metabolites of drug candidates emphasized the need for such a method that robustly quantifies metabolic products  
298 without resort to perhaps unpredicted and unavailable internal standards (27).

299  
300 The reproduced trace in Figure 5 is significantly correlated (Spearman rank correlation: 0.943). The differences  
301 between the repeats average 0.6% with an RMS difference of 5.4%, primarily contributed by the 5 minute point. The  
302 individual error bars represent 1 SD uncertainty due to both sample and carrier measurement precision and, as such,  
303 should overlap at only two thirds of the data points. Only one of six replications do not overlap, however, showing a  
304 better than normal repeatability for the procedure. The non-overlapping elution fraction is at the start of a metabolite  
305 peak where very slight changes in fraction definition have comparatively larger effects. Quantitation is in the attomole  
306 range for this 2% labeled compound, allowing mass quantitation into the tens of femtograms.

307



308

309 *Figure 5. Repeatability in AMS analysis of metabolite profiles by UPLC is shown for two LC runs completed on*  
 310 *separate days. The mass of equivalent drug was determined from the <sup>14</sup>C concentration, the specific labeling of the*  
 311 *compound, and the molecular weight. Error bars represent propagated errors due to measurement of both the*  
 312 *fraction and the added carrier compound.*

313

314 The repeatability studies at high <sup>14</sup>C concentrations (Figure 3) and low <sup>14</sup>C concentrations (Figure 5) suggest that  
 315 AMS measurements are highly reproducible (≤1%) and that complete process reproducibility was within 5%.

316

#### 317 **2.4.2 Precision**

318 AMS precision of a single measurement follows closely the Poisson uncertainty of the inverse of the square root of  
 319 the total <sup>14</sup>C counts (28). A single measurement is made to almost any desired precision by obtaining sufficient  
 320 counts, but the AMS measurement process includes sample aliquoting and conversion as well as spectrometric  
 321 quantification. Figure 1 demonstrated stability, and thus a precision limit, in measuring multiple aliquots of sera to  
 322 about 2.5%, in agreement with the long term AMS repeatability for an external standard material shown in Figure 4.  
 323 Two other materials are frequently measured and provide other data to characterize measurement precisions.

324

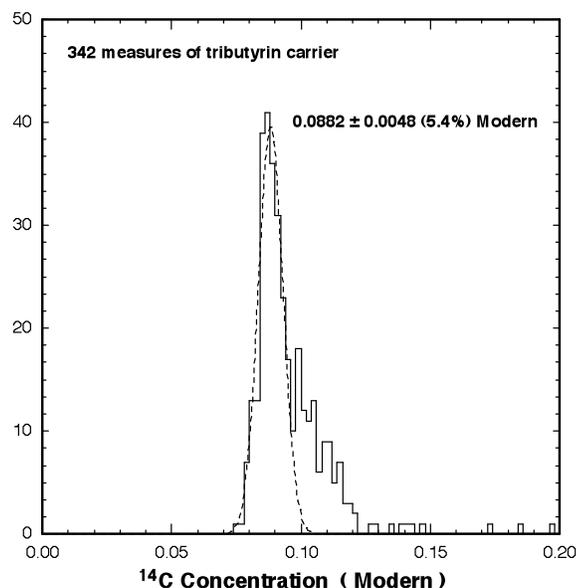
325 Biochemical isolations often produce samples that have very low carbon content. The preferred method of presenting  
 326 the sample in the AMS spectrometer is as a stable reduced carbon solid). A high-throughput process was described  
 327 and patented for this process (29) which works best with a minimum of 350 μg of carbon. Biochemical isolates of  
 328 even smaller mass are measured in a “carrier-added” mode in which known amounts of a well characterized

329 compound are added to the sample prior to processing to graphite. Tributyrin is a non-polar, viscous fluid  
330 hydrocarbon at room temperature with a low vapor pressure that is highly suitable for such a carrier. It is 60% carbon  
331 and contains no nitrogen, easing the condensation of the CO<sub>2</sub> from the sample's combustion. Typically, a 1 µl glass  
332 capillary of tributyrin (0.615 mg C) is added to samples of low mass. Multiple "blank" samples of the carrier are also  
333 processed and measured with the sample series. When the background materials are low in <sup>14</sup>C, the Poisson nature of  
334 AMS counting allows an improvement in background precision, and lower LLOQ, through longer measurement  
335 periods. <sup>14</sup>C-free materials are undesirable in this purpose, however, because measuring trustworthy background  
336 errors requires very long measurements, and the inadvertent incorporation of carbon "contamination" is undetectable  
337 (13).

338

339 Several hundred measures of the tributyrin carrier had a normal distribution about 8.8% Modern with an enhanced tail  
340 toward higher values (Figure 6). Very small contaminations of adsorbed Modern CO<sub>2</sub> on sample vessels can add <sup>14</sup>C  
341 to produce such a tail. The normal distribution is fit to the primary peak of the frequency distribution and indicates a  
342 precision of 5.4% CV. This precision is dominated by counting statistics, because often only 1000 counts were  
343 obtained for each measurement in these low <sup>14</sup>C samples, predicting a precision no better than 3%. Higher counting  
344 precision is possible for low <sup>14</sup>C samples through longer count times, at a cost of decreased daily throughput. In  
345 routine operation, 15-20 samples are measured per hour, but series of carrier-added fractions decrease this to about  
346 10-15 per hour.

347



348

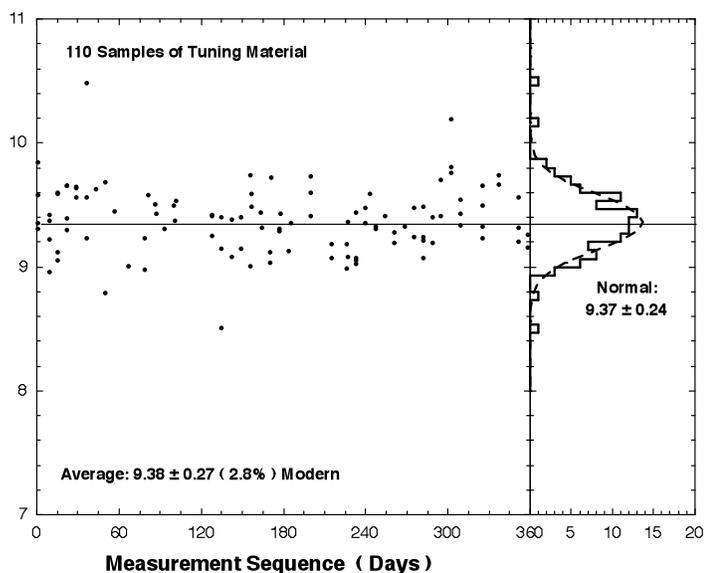
349 *Figure 6. The frequency distribution of a 0.1 Modern carrier compound has a standard deviation of 5.4%, due*  
 350 *primarily to counting statistics limited by impatient operators. A tail toward high values indicates occasional*  
 351 *contamination with higher <sup>14</sup>C.*

352

353 A 9 Modern material was used for assuring that the spectrometer is adjusted for optimal 14C transmission. A year's  
 354 worth of measurements is shown in Figure 7 for this homogenized leaf litter from an “<sup>14</sup>C enhanced” forest that has  
 355 been counted by several decay counters in environmental research laboratories and by LLNL AMS (30). The  
 356 consensus value was found to be 9.01 Modern, but AMS routinely gives a 3% higher value. More than 100  
 357 measurements over a period of one year show a near normal distribution with a CV of 2.6%. There is no significant  
 358 time trend in the data ( $r^2 = 0.0005$ ).

359

360 Routine AMS measurements for biochemical analyses thus have an overall process precision of 2-5%, including  
 361 sample aliquoting to isotope ratio determination, as presently quantified. .



362

363 *Figure 7. The main plot shows the measured <sup>14</sup>C concentrations versus time of measurements over a year's period for*  
 364 *a "hot" international comparison material. The measurements have a standard deviation of 2.8% and are well*  
 365 *represented by a Gaussian distribution function with a 2.6% width.*

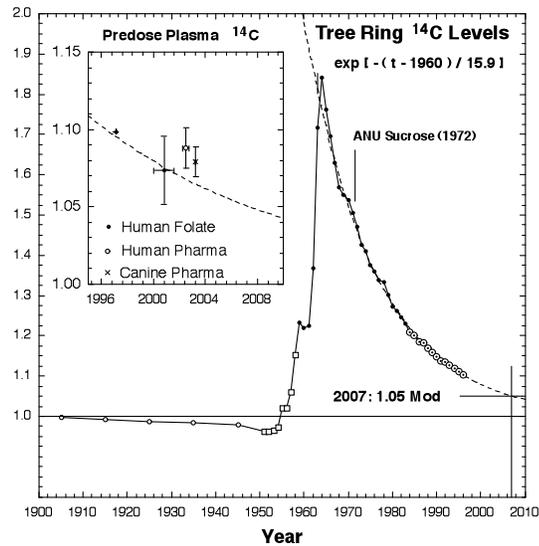
366

367

368 **2.4.3 Accuracy**

369 The precision, linearity, stability, and specificity shown above for AMS already imply a degree of accuracy within the  
 370 3% limits of measurement precisions. Determination of AMS accuracy is not needed across the entire range, although  
 371 multiple trusted calibration materials are being developed. We discuss accuracy in terms of common materials of  
 372 knowable isotope concentration and of direct AMS comparisons with accepted LSC measured samples. Accuracy of  
 373 AMS quantitation to much better than 1% is well documented in the range of carbon dating by quantifying countable  
 374 tree rings or annual sediment layers used in calibrated radiocarbon dating (22).

375



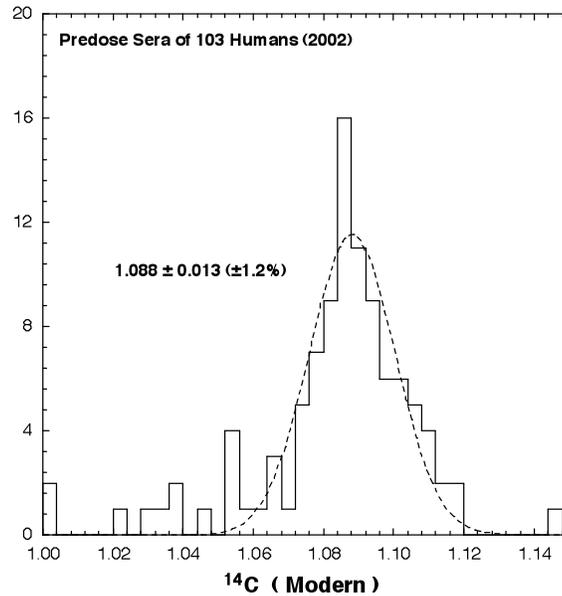
376

377 *Figure 8. Atmospheric <sup>14</sup>C concentration as reflected in tree rings and other plant material. The “bomb spike” of*  
 378 *1963 has become an essential tracing tool for carbon movement throughout oceans, soils, and humans. Predose*  
 379 *samples of subject plasma will closely correlate with the atmospheric concentration.*

380

381 A NIST-sourced standard of known <sup>14</sup>C concentration is used as primary standards in <sup>14</sup>C dating and for normalizing  
 382 most AMS isotope ratios (SRM- 4990 B,C). The average atmospheric <sup>14</sup>C concentration is present in recently grown  
 383 plant materials and reflected in the pre-dose <sup>14</sup>C concentrations of rapidly replaced constituents of living animal hosts  
 384 and human subjects, such as blood plasma. This concentration is known and determined with high confidence. <sup>14</sup>C  
 385 concentrations in living subjects closely follows the atmospheric concentrations as recorded in tree rings or annual  
 386 growth products (seeds or leaves). <sup>14</sup>C is so rapidly equilibrated throughout the atmosphere (a few weeks) that the  
 387 differences in <sup>14</sup>C concentration among peoples throughout the world are minimal. The <sup>14</sup>C concentration of the  
 388 atmosphere and living biosphere in 1900 was right at the NIST-traceable concentration called “Modern” (Figure 8).  
 389 The increased industrial use of fossil fuels in the 20<sup>th</sup> century contributed to a decrease in the atmospheric <sup>14</sup>C isotopic  
 390 concentration, which was greatest during the intense industrial activity of World War II, as plotted in Figure 8. At that  
 391 point, the testing of nuclear explosions above ground produced large amounts of <sup>14</sup>C through the interactions of  
 392 released neutrons with ubiquitous nitrogen. This trend reached a maximum in 1962, when multiple weapons tested in  
 393 the upper atmosphere almost doubled the amount of <sup>14</sup>C throughout the world’s air. A treaty banned such tests and  
 394 allowed the natural exchange of CO<sub>2</sub> between the atmosphere and the oceans to lower the <sup>14</sup>C concentration with an

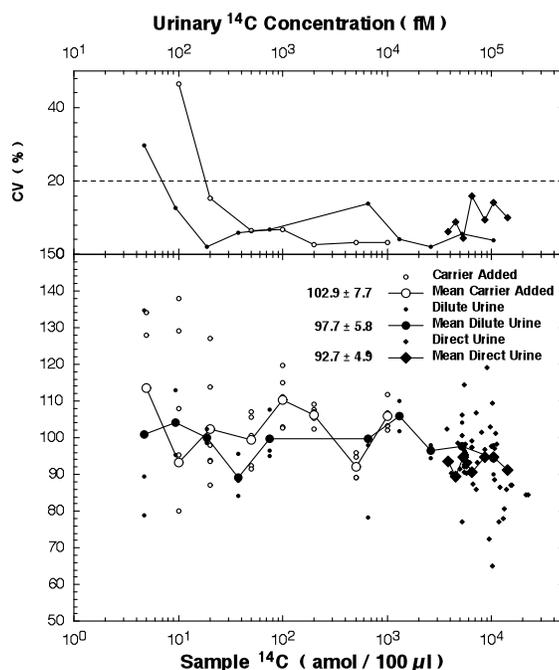
395 apparent mean life of 16 years over the past four decades. A contemporary human should have 1.04 Modern (in 2009)  
396 blood, urine, and sloughed epithelial cells, but if test animals (or subjects!) are eating “old” feed, they may have more  
397  $^{14}\text{C}$  than is expected. Certain tissues or isolates from the body are highly retained through slow or incomplete  
398 replacement, and their lifetimes are estimated from the high levels of  $^{14}\text{C}$  concentration reflecting production and  
399 retention from a period of higher atmospheric  $^{14}\text{C}$  over the past 40 years (31); (32); (33); (34).  
400



401  
402 *Figure 9. A frequency distribution plot of  $^{14}\text{C}$  concentration in blood serum for 103 humans during late 2002 shows a*  
403 *peak at the expected 1.08 Modern with a 1.2% standard deviation in the Gaussian fit.*  
404

405 Figure 9 shows the frequency distribution of  $^{14}\text{C}$  concentrations in 103 predose human sera taken in 2002 from 3  
406 different clinical sites. Slight natural variation in human  $^{14}\text{C}$  levels is expected, and the collection and processing of  
407 samples from multiple clinical sites may introduce further variability (see Contamination, below). The distribution tail  
408 toward lower values may well represent chemical or physical incorporation of plastic from the collection tubes.  
409 However, the main body of these measurements clusters around 1.08 Modern (open circle, Figure 8 inset) and has a  
410 near-normal distribution with a 1.2% CV. The CV of the average of all 103 measurements is 2.4%, although the entire  
411 data set is not normally distributed. The high peak at the mean value indicates an even tighter underlying population  
412 among the data. Other predose plasma data (solid circle, Figure 8 inset) are taken from a study of folate  
413 pharmacokinetics in 13 subjects (both genders, mean age: 24) done in 1999-2001 for which the average value was  
19

414  $1.074 \pm 0.022$  Modern (35). One subject was studied in a feasibility experiment for the folate study in 1997 and shows  
 415 the expected higher starting concentration. Finally, 6 dogs were used in a microdose feasibility study of a candidate  
 416 compound in early 2003 in which an average predose plasma concentration of  $1.0792 \pm 0.0098$  Modern was measured  
 417 (36). These samples are all within the expected 2.5% AMS precision of the atmospheric  $^{14}\text{C}$  at the time of collection,  
 418 indicating that accuracy of AMS quantitation is within the already established precision for moderate amounts of  
 419 tracer label.  
 420



421  
 422 *Figure 10. The AMS recovery fractions of labeled compounds in both a biological fluid (urine) and a carrier*  
 423 *compound are shown in the lower frame which includes direct comparison of LSC data with the AMS recovery*  
 424 *(diamonds) and the dilution of a “hot” urine into further urine (closed circles). AMS recovery of  $^{14}\text{C}$ -AZT in carrier*  
 425 *compound is shown in open circles. The upper frame shows the standard deviation of these data sets as a function as*  
 426 *assayed  $^{14}\text{C}$ .*

427  
 428 A direct comparison of LSC and AMS counting of high  $^{14}\text{C}$  urine samples (90 - 200 Modern) without using any  
 429 dilution techniques is shown above in Figure 2 and plotted as isotope label recovery in Figure 10. Accuracy to  $93 \pm 5\%$   
 430 is shown between AMS and the typically accepted LSC data for 100  $\mu\text{l}$  urine samples containing 2 to 20 femtomoles  
 431 of the isotopic label. We already showed an AMS repeatability in this range of about 1% and expect a total precision

432 of 3-5% for the AMS quantitations, but there are multiple sources for differences between the methods. A volume of  
433 urine is combined with scintillant for an LSC measurement, and the product allowed to sit in darkness for several  
434 hours to reduce chemical luminescence before counting is done. These samples were carefully counted for long times  
435 using quench and deadtime corrections. AMS samples began with smaller volumes of urine which were vacuum  
436 centrifuged to dryness before combustion to CO<sub>2</sub> and reduction to fullerene powder for AMS measurement. The  
437 comparison of LSC to AMS requires knowledge of the highly variable carbon concentration of urine, since AMS  
438 provides a <sup>14</sup>C isotope ratio and LSC measures a <sup>14</sup>C content in a volume. Obtaining accurate carbon contents in yet a  
439 third aliquot of the urine collection was the most demanding procedure of the comparison. Carbon contents of human  
440 urine were found to have a mean at 4 mg/ml, with a 50% spread. The 95% confidence interval spanned 1.5 to 10.5  
441 mg/ml. Thus, AMS accurately reflected the same level of <sup>14</sup>C as LSC in these samples, but the multiple sources of  
442 uncertainty leave a large scatter in the data.

443

444 The dilution series in urine displayed a 98± 6% accuracy for AMS recovered quantitation in Figure 10, while spiked  
445 amounts of <sup>14</sup>C-AZT in tributyrin and tricine carrier aliquots showed a 103±8% accuracy, both from 5 amol to 20 fmol  
446 of tracer label. These data are consistent with a 100% accuracy reflected in recovered signal within the measurement  
447 errors across a range of 10<sup>4</sup> in tracer signal.

448

449 Overall, the agreement of predose samples with atmospheric concentrations to within a few percent, the 3%  
450 agreement of the 9 Modern material with a, perhaps suspect, consensus level, the 2-3% difference between diluted  
451 spike and AMS determinations on samples up to 200 Modern, and the published accuracy of AMS for carbon dating  
452 to better than 1% demonstrate an accuracy that is better than stated AMS precisions across more than four orders of  
453 magnitude for biochemical AMS measurements.

454

### 455 **3. SENSITIVITY (LOD, LLOQ)**

456

457 Data are confidently accepted for the intended purpose if they fall above the limit of detection (LOD) and if they are  
458 quantified above the lower limit of quantitation (LLOQ). Two methods can define these limits, one statistical and the

459 other empirical. Multiple measures of the same sample in an AMS provide a Gaussian distribution to a high degree of  
460 precision (28) and this paper shows that multiple measures of materials over long periods of time produce Gaussian  
461 distributions (Figures 4, 6, 7, 9) over a variety of  $^{14}\text{C}$  concentrations. Statistical methods that assume normal  
462 distributions are therefore applicable to the AMS measurement process. AMS, unlike most bioanalytical methods,  
463 fulfills the homoscedastic assumption that precision of measurement is independent of the analyte concentration.  
464 Homoscedasticity arises from the Poissonian statistics of the analysis and from the nature of the background, which is  
465 true  $^{14}\text{C}$  found in background samples due to natural abundance or to accidental contaminations. These backgrounds  
466 are measured to the same desired precision as a low-signal sample, supporting definitions of LOD and LLOQ as 3.3  
467 and 10 times the SD found from a group of proper background samples (3). Biochemical AMS measurements  
468 generally have one of two possible backgrounds: the natural  $^{14}\text{C}$  present in all living materials, or the  $^{14}\text{C}$  in a diluent  
469 or other compound introduced in sample definition and processing.

470

471 Figure 3 shows that a preferred carrier compound is 9% Modern carbon and has a standard deviation in routine  
472 analyses ( $\approx 1000$  counts  $^{14}\text{C}$ ) of 0.005 Modern from the 1  $\mu\text{l}$  aliquot, equivalent to 300 zeptomoles  $^{14}\text{C}$ . The statistical  
473 LLOQ for small amounts of biochemical isolates that are combined with this carrier is then 3 amol, or about 7.5 fg of  
474 a 250 gram compound that has a 10% isomolar fraction (specific activity = 6.42 Ci/mol). The LOD is 1 amol. Figure  
475 9 shows that people from multiple clinical sites over a multimonth period have a natural  $^{14}\text{C}$  concentration quantified  
476 to 1.2% (CV). This natural level is approximately 100 amol of  $^{14}\text{C}$  per mg of carbon, and an LLOQ of 10 times the  
477 1.2% CV of this background is about 12%, or about 12 amol in a mg of carbon (available from  $\approx 20$   $\mu\text{l}$  of plasma or  
478 200  $\mu\text{l}$  of urine). The 250 gram compound with 10%  $^{14}\text{C}$  labeling is thus quantified to a statistical LLOQ of 30 fg in  
479 the sample.

480

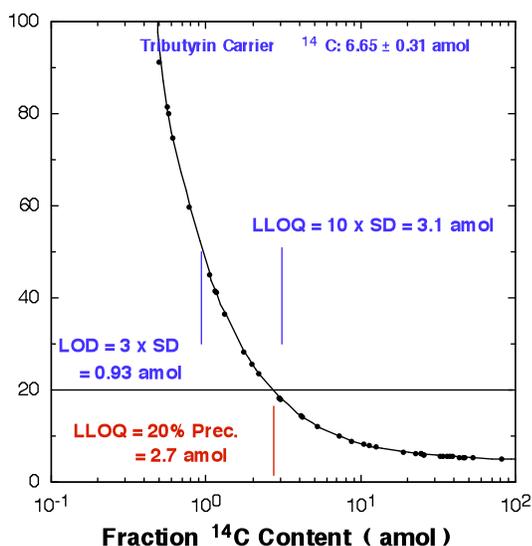
481 AMS measurements involve more than just isotope quantification: sample collection, storage, transport, separation,  
482 dilution, conversion, etc. Figures 4 and 5 show the distributions of multiple measures of  $^{14}\text{C}$  in a high and a low level  
483 material. These distributions (as with the human sera in Figure 9) show some non-Gaussian behavior, particularly in  
484 the distributions' tails. Empirically derived LLOQs are compared with statistically derived LLOQ concentrations to  
485 investigate the magnitude of contributions from this variety of procedures and matrices. Figure 10 shows the AMS

486 signal recovery plot for isotopic labels in both urine and carrier compounds along with the standard deviations of  
487 repeated samples at specific dilution or spike levels of grouped measurements in the direct LSC-AMS comparison  
488 measurements. These “recovery” percentages are plotted versus the assumed correct level in the top graph of the  
489 figure, providing an empirical way to define the LLOQ. If an error of 20% is acceptable in the biochemical  
490 measurement, these precisions of repeated measurements yield an LLOQ of 7 amol  $^{14}\text{C}$  in the 100  $\mu\text{l}$  urine samples.  
491 The unspiked urine dilutant was measured 5 times with an average of  $1.057 \pm 0.019$  (1.8%) Modern, suggesting a  
492 statistical LLOQ of 15 amol ( $10 \times 0.019$  Modern  $\times 97.8$  amol/mgC  $\times 4$  mg/ml  $\times 0.2$  ml). The experimental LLOQ is  
493 equivalent to about 5 times the error in the background in this case, less than the statistical prediction but of the  
494 expected order of magnitude.

495

496 Figure 11 shows the measurement precision versus  $^{14}\text{C}$  content of a series of LC fractions collected in a study of a  
497 drug’s metabolite profile. Four samples of the carrier averaged  $0.111 \pm 0.005$  ( $\pm 3.4\%$  CV) Modern. Ten times the SD  
498 of this measurement suggests an LLOQ of 3.1 amol  $^{14}\text{C}$  ( $10 \times 0.011$  Modern  $\times 97.8$  amol/mgC  $\times 0.615$  mgC). The  
499 error assigned to each fraction measurement is the uncertainty in this carrier background and an assumed uncertainty  
500 in the fraction measured (based on the 5.4% reproducibility shown in Figure 4) added in quadrature. We see that the  
501 measurement precision reaches 20% at 2.7 amol per fraction, again lower than the statistically predicted LLOQ.

502



503

504 *Figure 11. The assigned precision in the quantitation of LC eluent fractions is plotted against the amount of  $^{14}\text{C}$  in the*  
505 *fraction. The carrier carbon had an average value of  $0.111 \pm 0.005$  Modern (3.4%). An uncertainty in the single*  
506 *fraction measure was also assumed at 3.4%.*

507

508 The LLOQ of the entire AMS measurement process is apparently overestimated by the statistical definition of LLOQ  
509 at 10 times the background uncertainty, especially when that background is known to be stable and constant across all  
510 subjects or separation methods. Extraction of tracer compounds from biological fluids (SPE, LC, etc.) offers better  
511 LLOQ (5 versus 8 amol  $^{14}\text{C}$ ) over direct measurements of the fluid, but only if the background in carrier compounds is  
512 routinely measured to high precision. Multiple measurements are also required in separation procedures to show that  
513 the isolation technique is quantitative within the desired error, whereas neat biological fluids provide clean, robust  
514 measurements from simple combustion, reduction, and measurement of the sample. LLOQ's are best stated for the  
515 quantified tracer isotope,  $^{14}\text{C}$ , while individual analyses can convert these to grams of compound by knowing  
516 molecular weight and the isomolar fraction of the dosed component.

517

518

#### 519 **4. CONTAMINATION**

520

521 AMS LLOQs extend to attomoles and micro-Bequerels, levels at which many biochemical tracing laboratories would  
522 appear havens of potential contamination for samples with  $^{14}\text{C}$ . There are procedures that reduce this to a manageable  
523 problem in all but the most extreme cases. Such contamination is easily recognized by unexpected outliers that are  
524 confirmed with Grubb's Test (extreme studentized deviate), as shown in Table 1. The confirmed outlier represents a  
525  $^{14}\text{C}$  contamination of about 250 amol or 0.04 Bq per ml urine in a series of predose collections, a level possibly added  
526 by fingerprints or aerosol contact with collection vessels in a clinic, facility, or laboratory that handles mega-Bq of  
527 labeled compounds.

528

529 The primary concept in preventing such problems is to establish practices in the laboratory that protect the sample to  
530 the best of one's knowledge ("Paranoid Lab Practice", PLP) – one changes gloves often, not to protect oneself or to

531 avoid transport of biochemicals, but to avoid contamination to the sample; use only new packs of plasticware from a  
 532 known source because the clean, empty vessel on the shelf may well have adsorbed volatile <sup>14</sup>C over the past week or  
 533 two; etc. The guiding principle of PLP is that every sample handler must positively know that the container,  
 534 instrument, solvent, buffer, etc. that is about to come in contact with the present sample is appropriately free of excess  
 535 <sup>14</sup>C. Surfaces (benchtops, sinks!!, freezer handles, etc.) that have excess <sup>14</sup>C confirmed by AMS swipe measurements  
 536 are usable through the epithelial principle, in which surfaces routinely shed a previous layer (aluminum foil, plastic  
 537 wrap, etc.) only to be covered by a fresh surface that the present handler knows to be clean. The single most  
 538 contaminating operation in the biochemical laboratory has been the drying of a sample prior to combustion by  
 539 lyophilization (not recommended) or vacuum centrifugation. Samples should not be left in these systems any longer  
 540 than necessary and certain systems are known to perform much better than others (Jouan steel and glass centrifuge  
 541 chamber coupled to a Unijet drying pump). The PLP practice is not overly onerous and has been repeatedly shown to  
 542 be the best way to obtain meaningful AMS measurements (Buchholz, et al., 2000 (24)).

543

544 **Table 1. Nine measures of predose urines.** Grubb's Test showed a significant outlier.

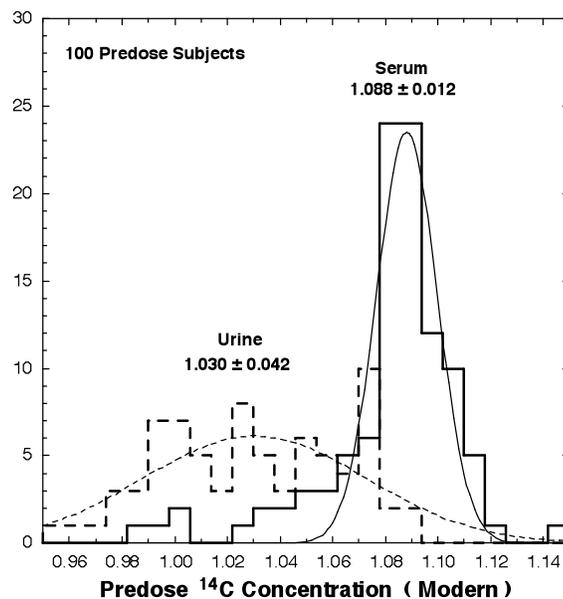
545

546

547

Value	Z	Significant Outlier?
1.084	0.364	
1.096	0.307	
1.097	0.306	
1.086	0.352	
1.095	0.313	
1.749	2.666	Significant outlier. P < 0.01
1.083	0.370	
1.085	0.357	

548



549

550 *Figure 12. Frequency distributions of  $^{14}\text{C}$  concentrations in serum and urine samples of predose human subjects at*  
 551 *multiple clinic sites show the expected tight distribution around the atmospheric level for the serum, but urine*  
 552 *concentrations are lower with large variability.*

553

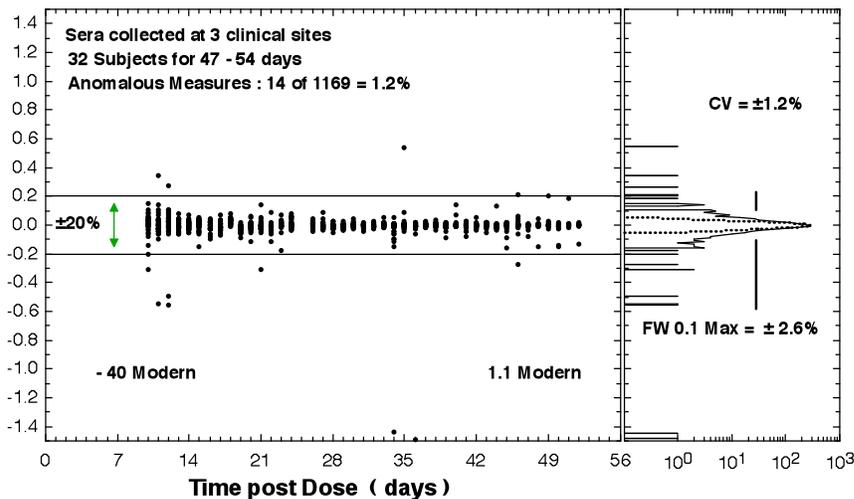
554 The rare tracer isotope is not the only possible contaminant. Any unplanned addition of carbon to a sample also  
 555 affects quantitation. One example showed how a non-volatile buffer was overlooked during LC quantitation (37).  
 556 Figure 12 shows the predose  $^{14}\text{C}$  content in human plasmas and urines collected from several clinical sites. The  
 557 plasmas have the expected natural level of  $^{14}\text{C}$  to about a 1% agreement among subjects, but the urine shows wide  $^{14}\text{C}$   
 558 levels centered at a lower mean, and not normally distributed. The low  $^{14}\text{C}$  ratios were traced to the plastic urine  
 559 collection bottles. A switch to alternate bottles produced urine concentrations that properly matched the plasma levels.  
 560 Leached plasticizers were not expected in collection products that had been in common use, nor would a benign fluid  
 561 like urine be expected to have special capability in leaching plastic. Traces of plastic powder from the injection  
 562 molding production process may have been at fault. It is imperative to collect multiple pre-dose samples to test the  
 563 entire clinic, laboratory, and measurement system. The uniformity of  $^{14}\text{C}$  within the population is, in fact, a strong

564 diagnostic for errors in proposed clinical procedures or for discovering subjects who may have volunteered in  
565 previous tracer studies.

566

567 A measure of the likelihood of sample contamination in a routine study was made by comparing individual data points  
568 with the interpolated value of neighboring points in the serum kinetic curves of a labeled compound in 32 human  
569 volunteers. The sera varied from 40 Modern at 10 days post dose down to near natural  $^{14}\text{C}$  levels (1.1 Modern) at 50  
570 days post dose. Figure 14 shows the percent deviation of each datum from the value interpolated from the four  
571 adjoining data. The overall distribution has a normal core (right panel) with a CV of 1.2% and a full width at 10%  
572 maximum of 2.6%, but the tails of the distribution are not normally distributed. Only 14 of the 1169 measures (1.2%)  
573 lie more than 20% from the established trend of each person's data. We note that a majority of the deviant values have  
574 lower  $^{14}\text{C}$  contents than expected, indicating again that the more likely contamination of a sample has been additional  
575 "dead" carbon from fossil sources (presumably from ubiquitous plastic labware). Only one measure had as much as  
576 50% more  $^{14}\text{C}$  than expected, showing that procedures are available to keep "hot" samples from contaminating the  
577 procedures and instrument.

578

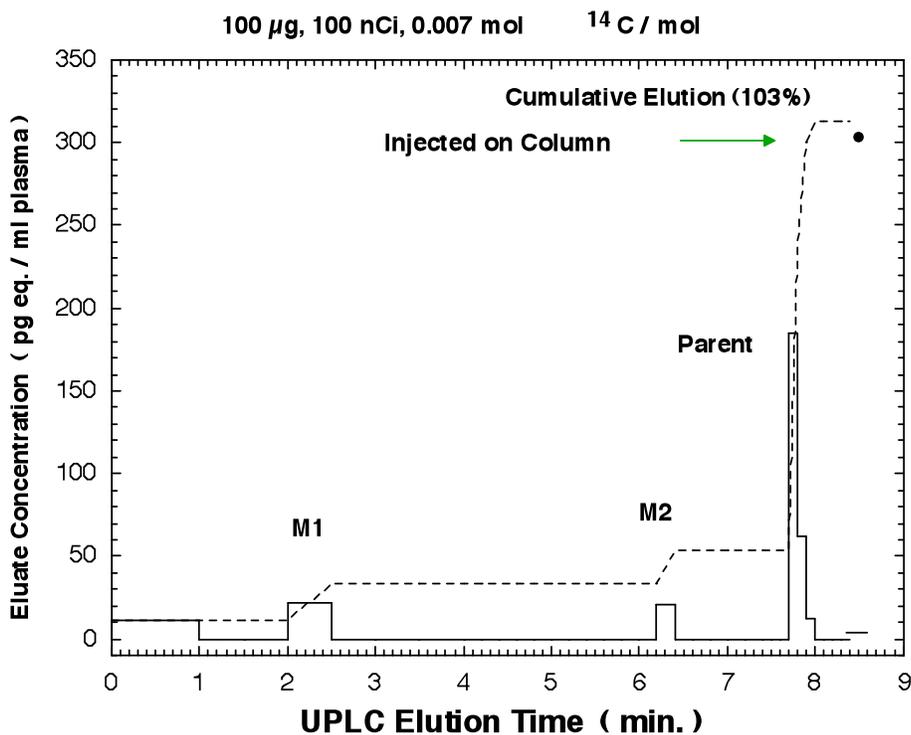


579

580 *Figure 14. A plot showing each data point's deviation from the interpolated value of neighboring data from the serum*  
581 *kinetic curves of 32 humans is shown as a function of the time post dose, in which the sera varied from 40 Modern to*  
582 *natural  $^{14}\text{C}$  levels. The right frame shows that the frequency distribution of the deviations has a normal core and*  
583 *enhanced tails.*

584

585



586

587 *Figure 15. Quantitation of UPLC eluent fractions of a parent compound and its metabolites are shown by the solid*  
588 *line. The cumulative sum of the quantified fractions (dashed line) is compared to the direct measure of the labeled*  
589 *compound in the injectate. The LLOQ was found as 10 times the uncertainty in the  $^{14}\text{C}$  concentration of the added*  
590 *carrier compound.*

591

## 592 5. QUANTITATIVE SUMMATION

593

594 The demonstrated linearity, reproducibility, precision, and accuracy of AMS quantitation support the concept of  
595 quantitative summation of fractionated samples, allowing for the quantitative comparison of different definition  
596 methods (38) and pointing toward any non-quantitative clinical or analytical procedures. A chemical or physical  
597 fractionation of a biological material should quantitatively reproduce the tracer concentration in the initial sample.  
598 One example is the comparison of the  $^{14}\text{C}$  content of a biological fluid and the sum of the fraction  $^{14}\text{C}$  contents from a  
599 chromatographic separation of the fluid. An example is shown in Figure 15, where the  $^{14}\text{C}$  content of a 20  $\mu\text{l}$  sample  
600 of the injectate is compared to the separated fractions and the sum of the fractions of a UPLC elution. Quantitative

601 summation such as this assures the analyst that no losses or contaminations occurred during chemical species  
602 definition. A similar robustness in AMS quantitation of gel-separated peptides led to a surprise conclusion that an  
603 ester was binding to an enzyme active site through covalent attachment to tyrosine instead of the expected lysine (39).

604

## 605 **6. DISCUSSION**

606 *AMS as a complement to other drug development technologies.*

607 AMS provides a highly sensitive, accurate and precise means of determining the  $^{14}\text{C}/\text{C}$  isotope ratio. Together with  
608 the careful measurement or control of the carbon inventory associated with a sample, this provides a highly sensitive  
609 means of measuring compound equivalents in a wide variety of matrices – both fluids and tissues, with great  
610 reliability and accuracy. This enhanced sensitivity (compared to radiometric means) can extend the range of  
611 observation and greatly decrease the radiological exposure, while improving quantitation for non-specific assays.  
612 When coupled to chemical separation, however, AMS becomes a very powerful pharmacokinetic and metabolism  
613 analytical tool for specific compounds.

614

615 Pharmacokinetic assays – especially from “microdoses” – of specific compounds are achieved by co-chromatography  
616 with an isonormal compound as a molecular carrier introduced in the extraction and/or chromatographic procedures.  
617 This allows monitoring the chromatographic separation by ultraviolet detection (for compounds with chromophores),  
618 while the quantitation relies upon the  $^{14}\text{C}$  isotopomer recovered post-column. This approach takes the best of all  
619 possible circumstances: high chemical concentration of the analyte is achieved by addition of the isonormal carrier,  
620 while sensitivity is achieved by the  $^{14}\text{C}$ -labeled analyte, now specific to the compound of interest, but isotopically  
621 distinct from the carrier addition.

622

623 For analysis of unknowns - most importantly metabolites - a quantitative summation approach should be used. This  
624 allows complete characterization and accounting of the  $^{14}\text{C}$ , so that the total recovery is known precisely and  
625 quantitatively through extraction, reconstitution and chromatography. In this way, the quality and certainty of result –  
626 the primary purpose of including  $^{14}\text{C}$  as a metabolism tool - is well satisfied. Recoveries greater than 90% of the

627 equivalents of  $^{14}\text{C}$  in the matrix are accounted for in the chromatograms, averaging  $97\pm 10\%$  ( $n=11$ ) in recent  
628 separations of both hydrophilic and hydrophobic parent compounds..

629

630 Of course, AMS is inherently non-specific, and any molecular identification has to be done with a companion  
631 technology, often by collecting multiple fractions of given chromatographic peak and determining molecular weights  
632 using molecular mass spectrometry, which is specific to mass to charge ratio. A strong advantage of AMS is that  
633 quantification, even independent of chemical identity, is quite reliable and can be used to determine which  
634 metabolites should be pursued and which are not of interest.

635

## 636 7. CONCLUSION

637

638 The measurements presented here were primarily obtained from the Biochemical AMS spectrometer at the Center  
639 for AMS at Lawrence Livermore National Laboratory. This spectrometer was carefully designed for maximal loss-  
640 free transmission (40) of ions produced by the uniquely intense LLNL ion source (41) from samples reduced to  
641 carbon with a high throughput process (29). However, most analytical properties described here arise from the  
642 fundamental isotope ratio nature of an AMS measurement on a homogeneous sample and will be applicable to most  
643 well designed and operated AMS spectrometers. In particular, the levels of specificity, stability, linearity,  
644 reproducibility, and accuracy are shared among spectrometers. The range, and LLOQ sensitivity may vary among  
645 instruments which have different levels of high energy mass and charge filtering and different capabilities for  
646 quantifying high count rates. The fundamental AMS precision depends on count totals due to Poisson statistics, hence  
647 on ion source output, but this is surmountable by longer measurement periods and is not a limiting factor as long as  
648 throughput is adequate.

649

650 The high  $\text{C}^-$  ion output of the LLNL ion source cannot be analyzed through the entire spectrometer because the intense  
651 ion current (100 - 500  $\mu\text{A}$ ) draws down the charging system for the high voltage. Instead, the isotope ratio of  $^{14}\text{C}/^{13}\text{C}$   
652 is quantified for the accelerated ions and is normalized back to the desired  $^{14}\text{C}/\text{C}$  ratio. This method has been shown to  
653 be precise and accurate to well under 1% (42). Other AMS spectrometers do quantify accelerated  $^{14}\text{C}/^{12}\text{C}$  and  $^{13}\text{C}/^{12}\text{C}$

654 ratios which provide further diagnostic capabilities at lower ion intensities, but do not add to any of the validated  
655 properties discussed here. .

656

657 Accelerator Mass Spectrometry is a uniquely quantitative tool with high specificity, stability, linearity, range,  
658 reproducibility, precision, accuracy and sensitivity. It is capable of repeated precise absolute quantitations of the  $^{14}\text{C}$   
659 content in a sample containing any  $^{14}\text{C}$ -labeled species without the need for internal standards or calibration curves.  
660 Robust AMS quantitation allows direct comparison of labeled compound quantities independent of processes used in  
661 definition and isolation. It frees the analytical chemist from frequent calibration and validation procedures that can be  
662 analyte-dependent. AMS sensitivity enables quantitative study of the ADME characteristics of drug candidate  
663 compounds directly in humans of all populations, including children, providing the analytical robustness to minimize  
664 subject numbers while increasing confidence in clinical ADME/PK-PD data. AMS is a superior quantitative tool but  
665 must be integrated with other analytical procedures (LC, LC/MS, NMR, etc) to provide the full picture of human  
666 response to chemical entities under study.

667

#### 668 **Analysis**

669

670 Linear regressions were performed with GraphPad InStat Software for Macintosh. Grubb tests for outliers were  
671 performed with Graphpad web QuickCalcs ([www.GraphPad.com](http://www.GraphPad.com)). Plots and functional fits were done with QuanSoft  
672 Pro Fit software for Macintosh ([www.quansoft.com](http://www.quansoft.com)).

673

#### 674 **Acknowledgements**

675

676 Some of this work was performed under the auspices of the U.S. Department of Energy by University of California,  
677 Lawrence Livermore National Laboratory under Contract W-7405-Eng-48. Colleagues at LLNL who performed work  
678 leading to the data shown here include Kurt Haack, Bruce Buchholz, and Darren Hillemonds. Portions of this work  
679 were supported by the NIH National Center for Research Resources at the Research Resource for Biomedical AMS  
680 (P41-RR013641). Many samples, data, and carbon analyses were done by or under contract with Procter & Gamble

681 Pharmaceuticals. Samples, data, and support in composing the manuscript were provided by Vitalea Science (Dr.  
682 Steve Dueker). Prepared by LLNL under Contract DE-AC52-07NA27344.

683

684 **Author Disclosures**

685

686 BDK is an employee of Vitalea Science (VS) which funds his authorship. BDK was an employee of Procter  
687 & Gamble Pharmaceuticals during much of this work, who sponsored many of the sample analyses  
688 referenced. TO is an employee of Lawrence Livermore National Laboratory and holds a patent licensed by  
689 VS but no other financial interests. JSV was an employee of Lawrence Livermore National Laboratory  
690 during much of this work but is now an unpaid advisor to VS and possesses equity in the Company which  
691 licenses several of his patents. No writing assistance was obtained from any other source. All affiliations  
692 with or financial involvement with any organization or entity with a financial interest in or financial conflict  
693 with the subject matter or materials discussed in their manuscript have been disclosed.

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697 **REFERENCES**

- 698 1. Viswanathan, C. T., Bansai, S., Booth, B., Destefano, A. J., Sailstad, J., Shah, V.P., Swann, P.G. and Weiner,  
699 R., Quantitative bioanalytical methods validation and implementation: best practices for chromatographic and  
700 ligand binding assays, *Pharm. Res.* Oct.24 (10), pp1962-1973, 2007.
- 701 2. U.S. Pharmacopeia, 23, pp. 1982-84, United States Pharmacopeial Convention, Inc., 1994.
- 702 3. ICH International Conference on Harmonisation, Draft Guideline on Validation of Analytical Procedures:  
703 Definitions and Terminology, Federal Register, Volume 60, pp. 11260, March 1, 1995.
- 704 4. FDA, Guideline for Submitting Samples and Analytical Data for Methods Validation, Food and Drug  
705 Administration, 1987.
- 706 5. FDA, Guidance for Industry: Q2B Validation of Analytical Procedures: Methodology CDER/CBER  
707 November 1996.
- 708 6. J.M. Green, A practical guide to analytical method validation, *Anal. Chem.* 68: pp. 305–309, 1996
- 709 7. Kutschera, W., Progress in isotope analysis at ultra-trace level by AMS. *International Journal of*  
710 *Mass Spectrometry*, 2005.
- 711 8. Scott, E.M., The Fourth International Radiocarbon Intercomparison (FIRI) Radiocarbon, 45: 135-150, 2003
- 712 9. Damon, P.E., Donahue, D.J., Gore, B.H., Hatheway, A.L., Jull, J.T., Linick, T.W., Sercel, P.J., Toolin, J.,  
713 Bronk, C.R., Hall, E.T., Hedges, R.E.M., Housley, R., Law, I.A., Perry, C., Bonani, G., Trumbore, S.,  
714 Woelfli, W. Davis, J.C., Proctor, I.D., Southon, J.R., Caffee, M.W., Heikkinen, D.W., Roberts, M.L., Moore,  
715 T.L., Tueteltaub, K.W., Nelson, D.E., Loyd, D.H. and Vogel, J.S., LLNL/UC AMS facility and research  
716 program, *Nucl. Insy and Meth in Phys. Res.* B53:269-272, 1990.
- 717 10. Turteltaub, K.W., Felton, J.S., Gledhill, B.L., Vogel, J.S., Southon, J.R., Caffee, M.W., Finkel, R.C., Nelson,  
718 D.E., Proctor, I.D. and Davis, J.C., Accelerator mass spectrometry in biomedical dosimetry: relationship  
719 between low-level exposure and covalent binding of heterocyclic amine carcinogens to DNA, *Proc. Natl.*  
720 *Acad. Sci.* Jul:87(14):5288-92, 1990. Shows the ability of AMS to reveal very low level adducts, and to use  
721 AMS with elaborate separation techniques to measure covalent adducts resulting from xenobiotic exposure.

- 722 11. Nelson, D.E., Mortan, R.E., Vogel, J.S., Southon, J.R. and Harrington, C.R., New dates on northern Yukon  
723 artifacts: Holocene, not Pleistocene, *Science* 232(4751):749-51, 1986.
- 724 12. Vogel, J.S., Accelerator mass spectrometry for quantitative in vivo tracing. *Biotechniques*. 2005 Jun;  
725 Suppl:25-9, 2005.
- 726 13. Vogel, J.S., and Love, A., Quantitating isotopic molecular labels with AMS. in Burlingame, AL, ed. *Meth.*,  
727 2005. **A key reference to AMS quantitation and calculations.**
- 728 14. Turteltaub, K.W., Vogel J.S., Bioanalytical applications of accelerator mass spectrometry for pharmaceutical  
729 research. *Curr Pharm Des.* 6(10):991-1007, 2000.
- 730 15. G. Lappin, R.C. Garner, Big physics, small doses: the use of AMS and PET in human microdosing of  
731 development drugs, *Nat. Rev. Drug Discov.* 2: 233. 523, 2003.
- 732 16. I.R. Wilding, J.A. Bell, Improved early clinical development through human microdosing studies, *Drug*  
733 *Discov. Today* 10: 890, 2005.
- 734 17. Stuiver, M. & Polach, H., Discussion: Reporting of <sup>14</sup>C Data, *Radiocarbon* 19, 355-363, 1977
- 735 18. Vogel, J.S. Rapid production of graphite without contamination for biomedical AMS *Radiocarbon* 34:344-  
736 350, 1992.
- 737 19. Freeman, S.P.H.T. and Vogel, J.S., Biomedical Accelerator Mass Spectrometry, *International Journal of Mass*  
738 *Spectrometry and Ion Process*, 1995.
- 739 20. Liberman RG, Tannenbaum SR, Hughey BJ, Shefer RE, Klinkowstein RE, Prakash C, Harriman SP, Skipper  
740 PL, An interface for direct analysis of (<sup>14</sup>)C in nonvolatile samples by accelerator mass spectrometry, *Anal*  
741 *Chem.* 76(2):328-34, 2004.
- 742 21. M.H. Choi, P.L. Skipper, J.S. Wishnok, S.R. Tannenbaum, Characterization of testosterone 11 beta-  
743 hydroxylation catalyzed by human liver microsomal cytochromes P450, *Drug Metab. Dispos.* 33: 714, 2005
- 744 22. Guildeson, T.P., Reimer, P.J. and Brown, T.A., Geoscience: Boon and Bane of radiocarbon dating, *Science*  
745 307 (5108): 362-4, 2005.
- 746 23. J.S. Vogel, J.R. Southon, D.E. Nelaon, and T.A. Brown, Performance of catalytically condensed carbon for  
747 use in accelerator mass spectrometry. *Nucl. Inst. and Meth B5*:289-293, 1984.
- 748 24. B.A. Buchholz , S.P.H.T. Freeman, K.W. Haack, J.S. Vogel, Tips and traps in the <sup>14</sup>C bio-AMS preparation

- 749 laboratory. Nuclear Instruments and Methods in Physics Research B 172: 404-408, 2000.
- 750 25. R.C. Garner, J. Barker, C. Flavell, J. V. Garner, M. Whattam, G. C. Young, N. Cussans, S. Jezequel and D.  
751 Leong A validation study comparing accelerator MS and liquid scintillation counting for analysis of <sup>14</sup>C-  
752 labelled drugs in plasma, urine and fecal extracts. Journal Pharmaceutical Biomedical Analysis 24: 197-209,  
753 2000.
- 754 26. Polach, H.A., A sucrose standard for <sup>14</sup>C dating. In Proceedings of the 9th International Conference on  
755 Radiocarbon; Berger, R., Suess, H., Eds.; UC Press: Berkeley/Los Angeles, CA; pp 115-24, 1979
- 756 27. FDA Guidance for Industry: Safety testing of drug metabolites. CDER February, 2008. The central  
757 regulatory guidance in the US for identifying and quantifying metabolites.
- 758 28. Vogel J.S., Ognibene T.J., Palmblad M., and Reimer P. Counting statistics and ion interval density in  
759 AMS. Radiocarbon. 46 (3): 1103-1109, 2004.
- 760 29. Ognibene T.J., Bench G., Vogel J.S., Peaslee G.F., Murov S. A high-throughput method for the  
761 conversion of CO<sub>2</sub> obtained from biochemical samples to graphite in septa-sealed vials for  
762 quantification of <sup>14</sup>C via accelerator mass spectrometry. Anal Chem. 75(9):2192-6, 2003. The  
763 reference procedure for conversion of carbon dioxide to graphite suitable for AMS measurement and  
764 efficient production.
- 765 30. Milton, G.M., Kramer, S.J., Milton, J.C.D., Interlaboratory Comparisons of C-14 Measurements in Milk and  
766 Vegetation, Radio carbon 40, 299-311, 1998.
- 767 31. Shapiro, S.D., Endicott, S.K., Province, M.A., Pierce, J.A., Campbell, E.J., Marked longevity of human lung  
768 parenchymal elastic fibers deduced from prevalence of D-aspartate and nuclear weapons-related radiocarbon.  
769 J Clin Invest. 87(5):1828-34, 1991.
- 770 32. Spalding, K., Bhardwaj, R.D., Buchholz, B., Druid, H. & Frisén, J. Cell 122, 133–143, 2005
- 771 33. Bhardwaj, R.D., Curtis, M.A., Spalding, K.L., Buchholz, B.A., Fink, D., Bjork-Eriksson, T., Nordborg, C.,  
772 Gage, F.H., Druid, H., Eriksson, P.S. and Frisen, J., Neocortical neurogenesis in humans is restricted to  
773 development, Proc. Natl Acad Sci USA Aug 15:103(33), pp12564-8, 2007.
- 774 34. Spalding, K., Arner, E., Westermark, P.O., Bernard, S., Buchholz, B.A., Bergmann, O., Blomqvist, L.,

- 775 Hoffstedt, J., Naslund, E., Britton, T., Concha, H., Hassan, M., Ryden, M., Frisen, J., Arner, P., Dynamics of  
776 fat cell turnover in humans, *Nature* May 8; 453(7192): 169, 2008.
- 777 35. Lin, Y., Dueker, S.R., Follett, J.R., Fadel, J.G., Arjomand, A., Schneider, P.D., Miller, J.W., Green, R.,  
778 Buchholz, B.A., Vogel, J.S., Phair, R.D., Clifford, A.J., Quantitation of in vivo human folate metabolism.  
779 *Am. J. Clin. Nutr.* 80(3): 680-691, 2004.
- 780 36. Sandhu, P., Vogel, J.S., Rose, M.J., Ubick, E.A., Brunner, J.E., Wallace, M.A., Adelsberger, J.K., Baker,  
781 M.P., Henderson, P.T., Pearson, P.G., Baillie, T.A., Evaluation of microdosing strategies for studies in  
782 preclinical drug development: demonstration of linear pharmacokinetics in dogs of a nucleoside analog over a  
783 50-fold dose range. *Drug Metab Dispos.* 32(11):1254-9, 2004.
- 784 37. Miyashita, M., Priestley, J.M., Buchholz, B.A., Lam, K.S., Lee, Y.M., Vogel, J.S., Hammock, B.D.,  
785 Attomole level protein sequencing by Edman degradation coupled with accelerator mass spectrometry. *Proc*  
786 *Nat Acad Sci* 98: 4403-4408, 2001.
- 787 38. Palmblad, M., Vogel, J.S., Quantitation of binding, recovery and desalting efficiency of peptides and proteins  
788 in solid phase extraction micropipette tips. *Journal of Chromatography B*, 814, 309–313, 2005.
- 789 39. J.S. Bennett, D.W. Hillegonds, B.A. Buchholz, E.S.C. Kwok, J.S. Vogel, T.H. Morton, Accelerator mass  
790 spectrometry for assaying irreversible covalent modification of an enzyme by acetoacetic ester, *International*  
791 *Journal of Mass Spectrometry* 179/180: 185–193, 2000.
- 792 40. T.J. Ognibene, T.A. Brown, J. P. Knezovich, M.L Roberts, J.R. Southon and J.S. Vogel, “Ion-optics  
793 calculations of the LLNL AMS system for biochemical C-14 measurements” 47-51, 2000.
- 794 41. Brown, T.A.; Roberts, M.L.; Southon, J.R. Ion-Source Modeling and Improved Performance of the CAMS  
795 High-Intensity Cs-Sputter Ion Source. *Nuclear Instruments & Methods in Physics Research Section B-Beam*  
796 *Interactions With Materials and Atoms*, pg 172, 2000.
- 797 42. Gorbarenko, S.A., Khusid, T.A., Basov, I.A., Oba, T., Southon, J.R., Koizumi, I., Glacial Holocene  
798 environment of the southeastern Okhotsk Sea: evidence from geochemical and palaeontological data.  
799 *Palaeogeography, Palaeoclimatology, Palaeoecology*, 2002.

800

801

801 Key definitions as sidebars.

802

803 1. Accelerator Mass Spectrometry (AMS). Isotope ratio mass spectrometry that measures the atom ratio of a rare  
804 isotope (carbon-14 in biomedical applications) as compared to the entire element present ( $^{14}\text{C}/\text{C}$ , here). It is  
805 chemically non-specific, and can be used to quantify specific molecular entities only when coupled experimentally to  
806 a suitable separation technology in such a manner that the excess carbon-14 measured above the matrix background  
807 is unique to that molecular entity.

808

809 2. Liquid Chromatography – Accelerator Mass Spectrometry (LC-AMS). When a labeled compound is isolated, and  
810 only the carbon-14 from that molecule is measured with AMS, then the concentration of that molecule can be  
811 determined with sensitivity and precision. Most commonly, either ultra performance liquid chromatography (UPLC)  
812 or high performance liquid chromatography (HPLC) is used to collect fractions that contain the analyte of interest  
813 which are then measured by AMS; this is known as LC-AMS.

814

815 3. Isomolar Fraction. The quantitative molar relationship between a compound and the carbon-14 it contains. A  
816 compound that is labeled so that 1 molecule in 100 bears a carbon-14 isotope would have a an isomolar fraction of  
817 0.01, this corresponds to a conventional specific activity of 0.624 mCi/mmol.

818

819 4. AMS Units of Measure. The results of an AMS measurement are expressed as an atom ratio of  $^{14}\text{C}/\text{C}$ . A special  
820 unit, derived from carbon dating is the unit of Modern, which has a value of  $1.118 \times 10^{-12} \text{ }^{14}\text{C}/\text{C}$ . Values less than 1  
821 Modern are often expressed as per cent Modern, while values greater than 1 – as would often be the case for a labeled  
822 compound - are often expressed as multiples of Modern; thus, a measurement result having a ratio of  $2.236 \times 10^{-12}$   
823  $^{14}\text{C}/\text{C}$  can be expressed as 2 Modern.

824

825 5. Equivalentents. The mass of labeled, parent compound that is represented by the measured quantity of  $^{14}\text{C}$  label, such  
826 as “nanogram equivalentents.” All AMS results arising from the introduction of a labeled compound, similar to  
827 radioactivity measurements, should be expressed as equivalentents unless the molecular entity giving rise to that

828 measurement is known. Only in cases where the molecular identity is known should the concentration be expressed  
829 as, for example, ng/mL, rather than ng-eq/mL.

830