

Precision and Accuracy in the Quantitative Analysis of Biological Samples by Accelerator Mass Spectrometry: Application in Microdose Absolute Bioavailability Studies

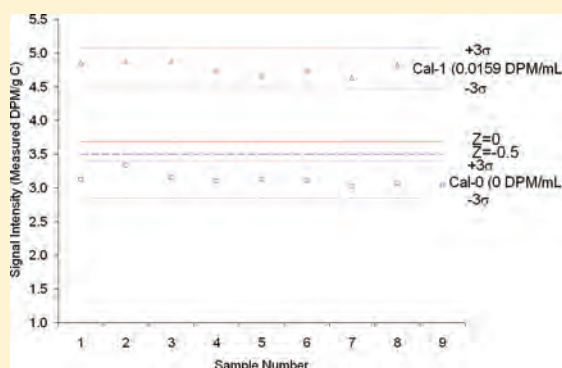
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S Supporting Information

ABSTRACT: Determination of the pharmacokinetics and absolute bioavailability of an experimental compound, SCH 900518, following a 89.7 nCi (100 μ g) intravenous (iv) dose of 14 C-SCH 900518 2 h post 200 mg oral administration of nonradiolabeled SCH 900518 to six healthy male subjects has been described. The plasma concentration of SCH 900518 was measured using a validated LC–MS/MS system, and accelerator mass spectrometry (AMS) was used for quantitative plasma 14 C-SCH 900518 concentration determination. Calibration standards and quality controls were included for every batch of sample analysis by AMS to ensure acceptable quality of the assay. Plasma 14 C-SCH 900518 concentrations were derived from the regression function established from the calibration standards, rather than directly from isotopic ratios from AMS measurement. The precision and accuracy of quality controls and calibration standards met the requirements of bioanalytical guidance (U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, Center for Veterinary Medicine. Guidance for Industry: Bioanalytical Method Validation (ucm070107), May 2001. <http://www.fda.gov/downloads/Drugs/Guidance-ComplianceRegulatoryInformation/Guidances/ucm070107.pdf>). The AMS measurement had a linear response range from 0.0159 to 9.07 dpm/mL for plasma 14 C-SCH 900518 concentrations. The CV and accuracy were 3.4–8.5% and 94–108% (82–119% for the lower limit of quantitation (LLOQ)), respectively, with a correlation coefficient of 0.9998. The absolute bioavailability was calculated from the dose-normalized area under the curve of iv and oral doses after the plasma concentrations were plotted vs the sampling time post oral dose. The mean absolute bioavailability of SCH 900518 was 40.8% (range 16.8–60.6%). The typical accuracy and standard deviation in AMS quantitative analysis of drugs from human plasma samples have been reported for the first time, and the impact of these parameters on quantitative analysis was further assessed using the Z factor. The use of the lowest achievable LLOQ_{Z=0} derived from statistical analysis of the control and low-concentration standards for AMS measurements is proposed in future studies.



Radioisotope (14 C and 3 H) labeled compounds are frequently used as tracers for pharmaceutical and medicinal studies to investigate the absorption, distribution, metabolism, and excretion of pharmaceuticals and nutritional supplements in animals and humans.¹ Samples from these studies are usually analyzed directly by liquid scintillation counting (LSC) for total radioactivity. HPLC coupled to in-line flow scintillation analysis or following fraction collection and off-line scintillation analysis is used to determine the metabolic profile or the concentration of the parent drug or metabolites.^{2–4} LSC determines the concentration of radioisotopes by detecting decay events of the radioisotope. LSC has a very poor efficiency for 14 C and 3 H nucleus counting due to the very slow frequency of decay events for these isotopes (e.g., the half-life of 14 C is 5730 years). When an analytical standard is available, liquid chromatography coupled

to tandem mass spectrometry (LC–MS/MS) is used for the detection and quantitation of selected analytes in biological samples because LC–MS/MS offers high sensitivity, excellent selectivity, automation, and superior accuracy in measurements. However, application of LC–MS/MS is limited in microdose studies, in which the dose administered is very low and the sensitivity of LC–MS/MS is often insufficient. Accelerator mass spectrometry (AMS) was developed in the 1970s for isotope ratio measurement, mainly for archeological carbon dating.⁵ Since the 1980s, AMS has been applied for biomedical studies

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to follow radioactive tracers, mainly ^{14}C , to study the fate of drugs, nutrient intake, and DNA/protein modifications.^{6–13}

The concept of microdose pharmacokinetics (PK) study was proposed around 2000.^{14–20} A microdose study involves an administration of $\leq 1/100\text{th}$ of the pharmacologically active dose, but not exceeding $100\text{ }\mu\text{g}$.^{21,22} This is a very attractive approach to obtain pharmacokinetic data early in the development program since this approach does not require a large amount of test article and an extensive preclinical safety package. Typically, two types of microdose studies in humans are conducted: a straightforward microdose PK study and an absolute bioavailability (AB) study. Due to concern of nonlinearity (dose versus exposure) in PK at microdose, straightforward microdose PK studies have not received widespread use.^{19,23} However, in AB studies the microdose is administered intravenously (iv) at the T_{max} of the therapeutic (pharmacologically active) nonradiolabeled oral dose to circumvent any potential nonlinear pharmacokinetic issues with the microdose.^{24,25} The iv microdose is given as radiolabeled ($\leq 100\text{ }\mu\text{g}$, $\leq 100\text{ nCi}$ of ^{14}C) to distinguish the iv-derived drug from the oral component. This paradigm is different from that of conventional AB studies, in which a two-period crossover clinical study design is often used.^{26–29} In these studies, the same subjects receive a nonradiolabeled oral or iv dose in one period, and after a washout period, the route of administration is switched. The microdose AB strategy has several advantages compared to the conventional crossover design. First, significant cost and time savings are achieved because formulation development for iv administration is not required, since such a small dose of drug can be dissolved in an administrable solvent (e.g., ethanol) and administered with saline. This is particularly beneficial for compounds of low solubility. Additionally, an iv formulation preclinical drug safety package is not required. Second, both iv and oral doses are administered at the same time; the PK data for both the iv and oral routes of administrations are obtained at the same total concentration at a given time. Therefore, the accuracy in absolute bioavailability and other PK parameters is expected to be higher than when administered separately in a two-period study.

Since LSC does not have the sensitivity to quantitate the drug level after a microdose administration, AMS was immediately identified as the tool of choice due to a sensitivity that is 3–6 orders of magnitude better than that of LSC and has been used for sample analysis of ^{14}C -labeled drugs. To measure an isotope ratio using AMS, the samples must be converted to graphite (for ^{14}C applications), TiH_2 (for ^3H applications), or another solid entity through laborious reduction procedures. However, the structure information of organic compounds is destroyed during the process. To measure a specific analyte concentration in a biological sample, the analyte must first be separated and purified from other compounds derived from the administered drug, such as metabolites. This is usually achieved by HPLC purification following extraction from the matrix. AMS has been used for microdose studies using ^{14}C -labeled drugs to measure compound concentrations in biological matrices. AMS does not measure the ^{14}C directly but measures the ratio of ^{14}C to ^{12}C or ^{14}C to ^{13}C . Equation 1

$$K = (R_{\text{sample}} - R_{\text{natural}})\Psi/L_{\text{mass}} \quad (1)$$

was often used to convert the AMS-measured isotope ratio to the analyte concentration (dpm/mL or ng equiv/mL), which was originally proposed by Vogel³⁰ and later modified/verified by

Salehpour³¹ and Lappin.³² In eq 1, K is the analyte concentration, R_{sample} is the isotope ratio ($^{14}\text{C}/^{12}\text{C}$) of the sample containing ^{14}C -analyte, R_{natural} is the isotope ratio of the natural background, Ψ is the carbon mass fraction in the sample (percentage of carbon in the sample), and L_{mass} is the specific activity of the ^{14}C -analyte expressed relative to the mass.

Standard samples prepared in ^{14}C -depleted tributyrin showed a 6 orders of magnitude linear AMS response (equivalent to approximately 0.005–200 dpm/mL) with high reproducibility in sample preparation and measurement.³³ A linear response ranging from 0.1 to 2.5 dpm/mL for spiked control plasma samples was also reported.³⁴ Chemical standards and machine/chemical blanks were typically used to calibrate the instrument performance. Limited or no control samples were included to calibrate the matrix effect, contaminations, and variations encountered during sample handling, processing, and measurement for ^{14}C concentration determination.^{32,35,36} The absolute AMS instrument sensitivity was taken for granted to be the lower limit of quantitation (LLOQ) of the AMS measurement of biological samples^{17,20,30} without appropriate considerations for the ^{14}C background from biological matrixes, contamination during sample handling, and reproducibility of sample processing and measurement. Alternatively, the LLOQ was arbitrarily defined as the concentration corresponding to 3 or 5 times the standard deviation (σ_c) of background signals (U_c) above the background ($U_c + 3\sigma_c$, or $U_c + 5\sigma_c$).^{31,37} The linearity of the AMS signal and analyte concentration has rarely been verified for any specific microdose PK study using AMS. Furthermore, a relatively large percentage of covariance ($\sim 30\%$) for biological samples was considered as routine.³⁷ Overall, there is a lack of understanding of quality and reliability achievable in quantitative analysis of biological samples by AMS. If these data are to be used in pharmacokinetic analysis for regulatory submissions, it is imperative to understand the operative precision and accuracy in AMS analysis of biological samples.

SCH 900518 is a Merck proprietary compound in development for treatment of hepatitis C virus (HCV) infections. A clinical study was conducted to obtain PK parameters and absolute bioavailability of the compound in healthy male subjects. In this study a therapeutic oral dose of 200 mg of unlabeled drug was followed by iv administration of a microdose (100 μg and 89.7 nCi) of ^{14}C -SCH 900518. A validated LC–MS/MS method was used for quantitative assessment of the unlabeled drug (oral component), and AMS was used for plasma ^{14}C -SCH 900518 concentration determination. To establish quality and control in the quantitative AMS plasma sample analysis, a set of calibration standards and triplicate quality controls (QCs) at three different concentration levels were prepared and included in the analysis of each batch of samples. The standards were prepared by spiking ^{14}C -SCH 900518 into blank human plasma. The ^{14}C -SCH 900518 plasma concentration in clinical samples was obtained from the regression functions derived from the AMS signal of the calibration standards. The accuracy and precision of both the calibration standards and QCs were examined for the acceptance of the assay. The dose-normalized area under the curve (AUC) values from iv (AMS) and oral (LC–MS/MS) plasma concentration–time plots were used to obtain the absolute bioavailability of the compound in humans.

A statistical analysis was conducted to differentiate the quantifiable ^{14}C signal from the administered drug from background ^{14}C signals using the Z factor.^{38,39} Although not widely used in bioanalytical assays, the Z factor has been used extensively in

Table 1. ^{14}C -SCH 900518 Recovery from Plasma Extraction Determined by AMS^a

sample name	AMS measurement (dpm/g of C)	^{14}C activity (dpm)	total dpm in 1.0 mL of plasma	^{14}C -SCH 900518 (dpm) in 1.0 mL of plasma	^{14}C -SCH 900518 recovery (%)
control plasma	3.302	0.000338	0.00264	0.000	na
control plasma	3.248	0.000281	0.00220	0.000	na
control plasma	3.252	0.000285	0.00223	0.000	na
QC-L-1	7.005	0.00424	0.0332	0.0308	136.4
QC-L-2	6.868	0.00410	0.0320	0.0297	131.4
QC-L-3	6.753	0.00398	0.0311	0.0287	127.2
QC-M-1	54.090	0.0539	0.421	0.419	79.1
QC-M-2	54.520	0.0544	0.425	0.422	79.8
QC-M-3	51.710	0.0514	0.402	0.399	75.4
QC-H-1	458.300	0.480	6.00	6.00	82.4
QC-H-2	440.400	0.461	5.77	5.77	79.2
QC-H-3	457.500	0.480	5.99	5.99	82.3

^aKey: column 4, $(1000 \times 250/400)(\text{column 3 divided by } 80 \text{ or } 50)$, 80 for the control and QC-L and QC-M samples and 50 for the QC-H samples; column 5, column 4 minus 0.00236 (mean ^{14}C in the control plasma); column 6, column 5 divided by 0.0226, 0.529, or 7.28 at the QC-L, QC-M, and QC-H concentrations, respectively. "na" means not applicable.

high-throughput screens to assess the quality of an assay in an attempt to predict if experimental conditions and measurements would be suitable in a high-throughput setting. The Z factor amalgamates the information of both the magnitude and the scale of the distributions of the signal and background and provides a better embodiment of the assay quality than the signal-to-noise or signal-to-background ratio alone. In this approach, the LLOQ could be defined as the concentration at which $Z \geq 0$. When $Z = 0$, there is a very high probability (>99.999998%) that the LLOQ of the system will be above the background signal window in a large experimental data set. The standard deviation in the lowest measurable analyte and the background signal can be determined from replicate blank and calibration standard analysis to determine $\text{LLOQ}_{Z=0}$. This approach could extend the LLOQ beyond the lowest calibration standard that can be prepared and would provide more accurate PK parameters. LLOQs determined on the basis of the standard bioanalytical guidance (lowest calibration standard that meets 5 times the noise level or a coefficient of variation (CV) and bias of <20%) and derived from statistical analysis were compared and used to calculate plasma PK parameters for SCH 900518. This investigation also provides de novo understanding of the achievable precision and accuracy in bioanalysis from human plasma samples by AMS.

EXPERIMENTAL SECTION

Materials. Methanol, formic acid, trifluoroacetic acid, and acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA). SCH 900518, ^{14}C -SCH 900518 (two lots, specific activity 58.5 and 0.897 $\mu\text{Ci}/\text{mg}$, respectively), the epimer SCH 783832, and closely eluting metabolites SCH 787443 and SCH 787444 were provided by Merck Research Laboratories (Kenilworth, NJ). Blank human plasma ($\text{K}_2\text{-EDTA}$ -treated) was purchased from Bioreclamation (Westbury, NY). Tributyrin was purchased from Sigma-Aldrich (St. Louis, MO).

Preparation of the Standard and QC Samples. Stock solutions of compound SCH 900518, epimer SCH 783832, and metabolites SCH 787443 and SCH 787444 were prepared at a concentration of 20 $\mu\text{g}/\text{mL}$ in 1:1 (v/v) acetonitrile/water and stored at -20°C . Nonradiolabeled SCH 900518 stock

solution was spiked into blank plasma to a concentration of 1.0 $\mu\text{g}/\text{mL}$. A stock solution of ^{14}C -SCH 900518 (specific activity 58.5 $\mu\text{Ci}/\text{mg}$) was prepared in 1:1 (v/v) acetonitrile/water at a concentration of 1.0 $\mu\text{Ci}/\text{mL}$. The calibration standard and quality control samples were prepared by spiking the ^{14}C -SCH 900518 stock or further diluted preparations into blank human plasma containing 1.0 $\mu\text{g}/\text{mL}$ nonradiolabeled SCH 900518 to concentrations ranging from 0.0159 to 9.07 dpm/mL. The ^{14}C -SCH 900518 concentrations of the calibration standards and QC samples were determined directly by AMS without isolation of the drug. L, M, and H in Table 1 designate low, medium, and high concentrations, respectively.

Clinical Study Design. This study was conducted in accordance with the principles of good clinical practice and was approved by the appropriate institutional review board and regulatory agency. All subjects provided written informed consent prior to initiation of the study procedures. Twenty-four healthy male subjects, screened in a separate study, provided blood samples to be used for determination of plasma background ^{14}C . Six healthy male subjects with a mean age of 40.5 years (ranged from 22 to 63 years) received 200 mg of SCH 900518 (2×100 mg) tablets on day 1 and an iv bolus dose of ^{14}C -SCH 900518 (100 μg , specific activity 0.897 $\mu\text{Ci}/\text{mg}$) in the arm 2 h post oral dose at a clinical facility (PRA International, Zuidlaren, The Netherlands). Blood samples (in tubes containing $\text{K}_2\text{-EDTA}$) were collected immediately before administration of the oral dose and at selected time points (0.5, 1, 2, 2.25, 2.5, 3, 4, 5, 6, 8, 10, 12, 16, 24, and 48 h) post oral dose administration. After centrifugation, the plasma samples were separated and divided into four equal fractions, two for AMS and two for LC-MS/MS analysis. The AMS sample portions were transferred to Accium BioSciences (Seattle, WA) for subsequent sample preparation and AMS measurement. The plasma concentrations of SCH 900518 and its epimer SCH 782832 were measured using a validated LC-MS/MS method, while the plasma ^{14}C -SCH 900518 concentrations were analyzed by AMS. The AUC was calculated following the noncompartment model using WinNonLin (version 5.2.1, Pharsight, Cary, NC) for each dose route. The absolute bioavailability (F) was calculated as the ratio of the dose-normalized AUC of the oral and iv

Table 2. HPLC Fraction ^{14}C -SCH 900518 Recovery from Plasma Extract Determined by AMS

sample name	AMS measurement (dpm/g of C)	total carbon ^a (mg)	^{14}C activity ^b (dpm)	natural background ^c (dpm/mL of plasma)	^{14}C -SCH 900518 activity in plasma extract (dpm/mL of plasma)	^{14}C -SCH 900518 activity recovered in HPLC fractions ^d (dpm/mL of plasma)	HPLC recovery (%)
control plasma	no data ^e						
control plasma	3.011	1.055	0.000193	0.000441		0.000	na ^f
control plasma	3.086	1.055	0.000688	0.000441		0.000	na
QC-L-1	8.010	1.055	0.0332	0.000441	0.0297	0.0327	110.0
QC-L-2	7.722	1.055	0.0313	0.000441	0.0297	0.0308	103.6
QC-L-3	no data						
QC-M-1	76.540	1.055	0.485	0.000441	0.414	0.485	117.2
QC-M-2	75.690	1.055	0.479	0.000441	0.414	0.479	115.8
QC-M-3	74.570	1.055	0.472	0.000441	0.414	0.472	114.0
QC-H-1	930.00	1.055	6.113	0.000441	5.92	6.11	103.2
QC-H-2	964.90	1.055	6.343	0.000441	5.92	6.34	107.1
QC-H-3	no data						

^a Total carbon content from carbon analysis. ^b Values prior to background subtraction. ^c Natural ^{14}C background determined by direct combustion of predose and control plasma samples. ^d Column 4 minus column 5. ^e "no data" indicates that no data were recorded in AMS analysis. ^f "na" means not applicable.

administrations, as described by the following equation:

$$F = [\text{AUC}_{\text{oral}}/D_{\text{oral}}]/[\text{AUC}_{\text{iv}}/D_{\text{iv}}] \quad (2)$$

where D_{oral} and AUC_{oral} are respectively the oral dose and oral AUC and D_{iv} and AUC_{iv} are the iv dose and iv AUC. SCH 900518 undergoes epimerization. The parent drug and the epimer coelute during HPLC fractionation and were measured together in this study because of difficulties in HPLC separation from each other. The reported absolute bioavailability represents the combined SCH 900518 and its epimer. The LC-MS/MS procedure used for analysis of the unlabeled orally administered drug had an LLOQ, an analytical range, a CV, and a bias of 6.11 ng/mL, 6.11–15300 ng/mL, 5.7–14.1%, and –0.6 to –5.5%, respectively.

Plasma Sample AMS Analysis. A 400 μL aliquot of a standard or clinical study plasma sample was transferred into a centrifuge tube and extracted twice with 1200 μL of plasma extraction solvent (acetonitrile containing nonradiolabeled SCH 900518, epimer SCH 782832, metabolites SCH 787443 and SCH 787444 at a concentration of 2 $\mu\text{g}/\text{mL}$ each, and 0.1% (v/v) trifluoroacetic acid). The nonradiolabeled reference analytes were added to ensure specificity during HPLC isolation of the parent drug and epimer as monitored by UV detection. The combined supernatant was evaporated to dryness under a nitrogen stream with heat controlled at 30 $^{\circ}\text{C}$. The dried plasma extracts were reconstituted with 250 μL of 7:3 methanol/water (v/v). The plasma extraction efficiency covering the drug concentration range in the study samples was determined by adding 25 μL of tributyrin to 80 μL (QC-L and QC-M) or 50 μL (QC-H) of plasma extraction supernatant prior to drying down, before graphitization. Petroleum-based tributyrin is a nonvolatile ^{14}C -depleted liquid and is used to compensate for the depletion of proteins (hence total C) during extraction of drug-derived material. The amount of ^{14}C -SCH 900518 recovered was corrected by subtracting the ^{14}C level in the control plasma extracts.

As indicated above, the dried plasma extracts were reconstituted with 250 μL of 7:3 (v/v) methanol/water. A 100 μL portion of the reconstituted sample was injected for chromatographic purification of the parent drug and epimer using a Shimadzu Prominence HPLC system (Shimadzu Scientific,

Columbia, MD) containing a Shimadzu SPD-10A UV detector and a Shimadzu FRC-10A fraction collector at a 1.0 mL/min flow rate. The parent drug SCH 900518 and its epimer SCH 782832 coeluted at approximately 17 min. This peak was separated by at least 1 min from those of all other human plasma metabolites. Eight 30 s fractions, which covered the whole analyte peak, were collected starting 2.0 min before the elution of SCH 900518. Four consecutive fractions corresponding to the center of the SCH 900518 peak as indicated by the UV absorbance monitored at 210 nm were pooled together for AMS analysis. A 150 μL aliquot of pooled HPLC fraction from each sample was transferred into separate quartz vials for AMS sample preparation. A known amount of carrier carbon (tributyrin, approximately 1.0 mg) was added to each quartz vial. The graphitization procedure, modeled after Ognibene,⁴⁰ converted the samples to graphite. A 500 kV double-stage Pelletron accelerator mass spectrometer equipped with a 40-sample ion source (National Electrostatics Corp, Middleton, WI) was used for the analysis at Accium BioSciences (Seattle, WA). The major components and operation of AMS instruments have been described in several recent reviews in detail.^{33,41–43} A typical AMS sample analysis batch consisted of 4 certified standards to normalize all measurements, 2 machine blanks (^{14}C -free graphite of natural origin) to assess the instrument background, 2 chemical blanks (samples prepared with ^{14}C -free substance) to characterize extraneous carbon introduced during sample preparation, if any, and up to 32 clinical study standard, QC, or unknown samples. The measured $^{14}\text{C}/^{12}\text{C}$ ratio was expressed as dpm/g of C.³³

RESULTS AND DISCUSSION

Plasma Extraction Efficiency and HPLC Recovery. The recovery of spiked ^{14}C -SCH 900518 through extraction and HPLC purification was assessed for each step. The plasma extraction efficiency covering the drug concentration range in the study samples was assessed using plasma standards at ^{14}C -SCH 900518 concentrations of 0.0, 0.0226, 0.529, and 7.28 dpm/mL following the procedure described in the Experimental Section but measured prior to the extracted supernatant being dried down. As demonstrated in Table 1, the extraction efficiency

(75–136%) was different at different concentrations. The recovery was artificially high (127–136%) at the lowest QC concentration, probably due to contamination, and was lower at higher QC concentrations. This persistent, systematic, and low-level contamination is likely from atmospheric ^{14}C . The low QC samples are more sensitive to this contamination, contributing to the high apparent recovery. The middle and high QC samples were also affected by this contamination, but this was not readily discerned in the final recovery because its effect was insignificant compared to the amount of ^{14}C already present at these concentrations. Although a mean background level radioactivity was subtracted (Table 1), the individual samples still showed high recovery potentially due to high variability in contamination in individual samples. This apparent inflated recovery at low concentrations and incomplete recovery at higher concentrations, if left uncorrected, would carry into the final quantitative results for analyte concentrations because recovery does not generally account for potential bias due to compound loss. The lower than 100% recovery in plasma extraction is not uncommon. As discussed later, this bias in the recovery can be corrected for by using calibration standards in each sample batch and deriving the analyte concentrations from the regression functions of the calibration curve. Background subtraction is not necessary.

To assess the recovery of ^{14}C -SCH 900518 through HPLC separation, supernatants from plasma extracts were dried and reconstituted as described in the Experimental Section. An aliquot of 100 μL of the reconstituted solution (250 μL) was injected into the HPLC instrument. A 25 μL tributyrin solution was added to 200 μL pooled HPLC fraction, the resulting solution was combusted and reduced, and the graphite was analyzed by AMS. The HPLC recovery (Table 2) ranged from 103% to 117%. This showed that relatively consistent and quantitative HPLC recovery was achieved for ^{14}C -SCH 900518 and its epimer at all concentrations studied.

Assessment of the Natural ^{14}C Background in Human Plasma and the Effect of Extraction and HPLC Isolation. AMS reports the $^{14}\text{C}/^{12}\text{C}$ isotope ratio in a sample. All living creatures have a natural ^{14}C abundance that is at equilibrium with the atmosphere and local environment. Human plasma background ^{14}C was determined to be 0.559 ± 0.005 dpm/mL by directly combusted and analyzed samples from 24 healthy male subjects screened in a different clinical study using AMS.⁴⁴ This value is consistent with repeated AMS measurement of one blank human plasma sample (0.567 ± 0.018 dpm/mL) reported previously.³⁴ After protein precipitation and HPLC isolation, the ^{14}C background in the HPLC eluent corresponding to the SCH 900518 retention time was reduced to 0.0000 ± 0.0010 dpm/mL.⁴⁴ The standard deviation for the background dropped 5-fold from 0.005 to 0.0010 dpm/mL. This improved the sensitivity and LLOQ of HPLC fractions relative to whole plasma.

Quantitative AMS Analysis. AMS sensitivity of 10^{-18} g of ^{14}C has been demonstrated for various standards.^{31,45} However, this superior instrument sensitivity is not readily achievable for biological samples since the relatively high abundance of carbon (e.g., plasma) contributes to the background signal. Analyte-specific ^{14}C -labeled calibration standards, QCs, or internal standards have rarely been included in quantitative AMS analysis of biological samples, perhaps due to historical precedent or the laborious sample preparation and the traditionally expensive nature of AMS analysis.³⁶ Measurement of $^{14}\text{C}/^{12}\text{C}$ ratios by AMS directly quantifies ^{14}C enrichment in the sample. Calculating the analyte concentration solely on the basis of AMS $^{14}\text{C}/^{12}\text{C}$

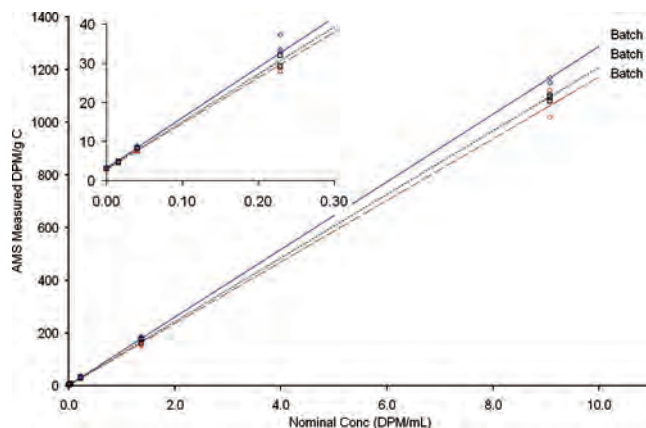


Figure 1. Linear regression fit with weight = $1/Y$ of calibration standards from three different batches for plasma ^{14}C -SCH 900518 analysis: batch 1 (open tilted squares and solid line), batch 2 (open squares and dotted line), batch 3 (open circles and dashed line).

ratios does not account for analyte losses that may have occurred during sample extraction or HPLC fractionation. These losses can be quantified and corrected for by using in-batch analyte-specific ^{14}C -labeled calibration standards and QCs along with traditional regression analysis to derive true drug concentrations in study samples.

Since AMS sample preparation converts all carbon in chemical structures to CO_2 during the oxidation step, an organic internal standard added to the sample becomes indistinguishable from the sample itself. Alternatively, nonradiolabeled internal standard can be employed and detected by UV absorbance to calibrate compound losses only at selected stages of sample processing.³² However, if calibration standards are included in each batch of plasma sample analysis, the concentrations can be determined from the regression analysis of calibration standard concentrations (Figure 1 and Table 3) and sample losses and systematic and low-level contaminations, and variability in the measurements can be compensated for all the way through AMS measurement (see the subsection “Precision and Accuracy in AMS Analysis of Biological Samples”). Furthermore, the introduction of QC samples provides an assessment of the precision and accuracy in AMS measurements.

In a recent proposal, Lappin et al. suggested analyte-specific AMS quantitation without the use of analyte-specific ^{14}C -labeled calibration standards, QCs, or internal standards.⁴⁶ Instead, extraction and LC recovery are proposed to be performed by an internal nonlabeled analyte quantified by LC/UV. The ^{14}C -labeled analyte measured by AMS would then be adjusted by the LC/UV method to determine the recovery of the nonlabeled analyte. This approach separates extraction and fractionation procedures from graphitization and AMS procedures. In so doing, LC/UV and its relatively poor sensitivity would be used to correct for loss of analyte present at concentrations only detectable by AMS. In contrast, our approach integrates the entire process and uses a single detection platform to determine both the recovery and analyte concentration. This approach was successfully applied to clinical studies and determined the precision and accuracy of measurements in biological samples.

Precision and Accuracy in AMS Analysis of Biological Samples. In this study, a set of calibration standards and triplicate QCs at three different levels were included in each batch of sample analysis. Variations in compound recovery in

Table 3. Precision and Accuracy in AMS Measurement of Plasma ^{14}C -SCH 900518 Concentration in Calibration Standards and QC Samples Derived from Regression Analysis of AMS Response and Calibration Standard Concentrations in One (Batch 101) of the Three Batches of Sample Analysis^a

sample name	nominal plasma ^{14}C -SCH 900518 concn (dpm/mL)	AMS measurement (dpm/g of C)	derived plasma ^{14}C -SCH 900518 concn (dpm/mL)	bias ^b (%)	CV (%)
Cal-1-1	0.0159	4.629	0.0130	−18.2	7.7
Cal-1-2	0.0159	4.810	0.0145	−8.8	7.7
Cal-2-2	0.0402	8.212	0.0427	6.1	0.4
Cal-2-3	0.0402	8.180	0.0424	5.5	0.4
Cal-3-1	0.229	29.240	0.2170	−5.1	7.1
Cal-3-2	0.229	31.994	0.2399	4.8	7.1
Cal-4-1	1.36	176.020	1.4341	5.2	4.0
Cal-4-2	1.36	176.480	1.4379	5.4	4.0
Cal-4-3	1.36	164.270	1.3367	−2.0	4.0
Cal-5-1	9.07	1093.800	9.0442	−0.3	0.9
Cal-5-2	9.07	1101.800	9.1106	0.4	0.9
Cal-5-3	9.07	1081.600	8.9431	−1.4	0.9
QC-1-2	0.0299	7.158	0.0339	13.6	3.4
QC-1-3	0.0299	7.357	0.0356	19.1	3.4
QC-2-1	0.531	64.930	0.5130	−3.4	2.7
QC-2-2	0.531	63.700	0.5028	−5.3	2.7
QC-2-3	0.531	67.150	0.5314	0.1	2.7
QC-3-1	6.70	855.500	7.0683	5.4	1.2
QC-3-2	6.70	857.500	7.0849	5.7	1.2
QC-3-3	6.70	874.100	7.2225	7.7	1.2
average ^c				1.7	3.4

^a Similar CVs (%) and accuracies (%) were obtained in the other two batches. The average number represents the mean data from all standard and QC sample analyses. ^b Bias (%) is the difference between the derived and nominal concentrations divided by the nominal concentration $\times 100$. ^c Mean data from all standard and QC sample analyses. $R^2 = 0.99980$, slope 120.6, and intercept 3.065.

clinical samples were largely compensated and controlled for by deriving the analyte concentrations from regression analysis of the calibration curve (Figure 1). The AMS responses from standards and QCs are summarized in Table 3. Measured and predicted ^{14}C -SCH 900518 plasma concentrations demonstrated a linear relationship in the range of 0.0159–9.07 dpm/mL using a 1/Y-weighted least-squares linear regression. The correlation coefficient was 0.9998, as determined by R^2 , indicating an excellent correlation between the AMS signal response and actual plasma concentration. The mean CV for standards and QCs was 3.4%. The highest CV obtained in all batches was 8.5% from the analysis of triplicate samples. The measurement accuracy (bias) ranged from 94% to 108%, with the exception of the lowest calibration standard and QC sample, where the measurement accuracy ranged from 82% to 119%. These results demonstrate for the first time that quantitative AMS measurement can meet the requirements of the bioanalytical acceptance criteria for plasma sample analysis. The regression analysis shown in Figure 1 demonstrates that the AMS response is linear up to 0 dpm/mL. The nonzero intercept indicates that a small amount of ^{14}C can be added to the actual radiocarbon present in all samples during sample handling and processing for AMS analysis potentially due to contamination from atmospheric $^{14}\text{CO}_2$. Most importantly, this contamination is corrected for by the intercept of the calibration curve (Figure 4 in the Supporting Information). To limit the number of samples that would need tedious sample processing and analysis, calibration standards at five concentration levels and triplicate QCs at three concentration levels were used per sample analysis protocol.

An Investigation into the LLOQ in AMS Measurements. As indicated by the widely accepted bioanalytical method validation guidance,⁴⁷ the LLOQ is defined as the concentration of the lowest calibration standard that has (1) 5 times the response of the blank control and (2) both precision (CV) and accuracy (bias) of less than 20%. However, the variation of the background level is not considered in the above definition. Application of these requirements to AMS biological sample analysis gives a higher LLOQ than the true LLOQ of the system due to the difficulty to accurately prepare calibration standards with precisely defined concentrations at levels close to what an AMS instrument can measure for the LLOQ. Therefore, in the absence of statistical evaluation of the variability in the control sample response, the LLOQ is limited to the lowest calibration standard, which is of much higher concentration than the true LLOQ of the system. We report an alternative approach to obtain a more meaningful and statistically significant LLOQ relevant to AMS data as discussed below.

A dimensionless parameter Z factor was proposed for such analysis and has been widely accepted to determine a statistically significant signal window for acceptance of biological assays.^{38,39} When a Gaussian distribution of a signal is assumed for the statistical analysis, the Z factor can be described by eq 3 and further simplified to eq 4

$$Z = 1 - 3(S_s + S_c)/\text{Abs}(U_s - U_c) \quad (3)$$

$$U_s = U_c + 3(S_s + S_c)/(1 - Z) \quad (4)$$

when $U_s \geq U_c$. U_s and U_c are the mean signals of the sample and

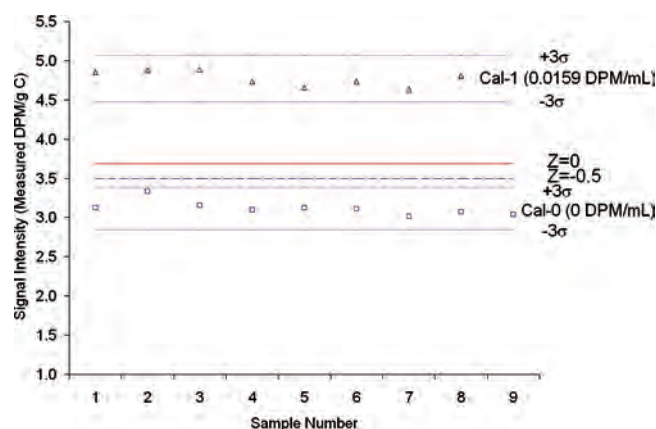
Table 4. AMS-Measured ^{14}C Radioactivity Level and the Standard Deviations of the Control Blank and the Lowest Calibration Standard Samples from Replicate Analyses

sample ID ^a	measured ^{14}C radioactivity (dpm/g of C)	mean ^{14}C radioactivity ^b (dpm/g of C)	std dev
101 Cal-0-1	3.121		
101 Cal-0-1	3.016		
101 Cal-0-2	3.332		
101 Cal-0-2	3.070		
101 Cal-0-3	3.153	3.119	0.09092
101 Cal-0-3	3.041		
104 Cal-0-1	3.099		
104 Cal-0-2	3.125		
104 Cal-0-3	3.110		
101 Cal-1-1	4.852		
101 Cal-1-1	4.629		
101 Cal-1-2	4.880		
101 Cal-1-2	4.810		
101 Cal-1-3	4.883	4.772	0.09896
104 Cal-1-1	4.733		
104 Cal-1-2	4.656		
104 Cal-1-3	4.736		

^a Cal-0 represents control samples with no ^{14}C standard added, and Cal-1 represents the lowest calibration standard. ^b The mean for Cal-0 is 0.0 dpm/mL, and the mean for Cal-1 is 0.0159 dpm/mL.

background, respectively, and S_s and S_c are the standard deviations from a limited set of samples, respectively. The Z factor value ranges from $-\infty$ to $+1$. As indicated,³⁸ the limit of detection can be defined as the concentration corresponding to $Z = -1$, where $U_s \approx U_c + 3\sigma_c$, assuming $S_s \approx S_c \approx \sigma_c$, where σ is the true standard deviation of the blank/control samples.

The LLOQ criteria defined by the bioanalysis guidance⁴⁷ could be interpreted as (1) a signal window big enough to distinguish the analyte or sample response (U_s) from the background or control sample response (U_c), $U_s \geq 5U_c$, and (2) the analyte having good enough precision and accuracy such that $S_s/U_s \leq 0.2$ ($\text{CV} \leq 20\%$). However, the variation in the measurement of the background signal was not considered in this definition. Since at the LLOQ level the analyte signal is close to the background, and the standard deviations represent the uncertainty due to the sample source, history, and processing and measurement, the standard deviations are expected to be similar for the backgrounds and low-concentration standards. Note that $U_s = 5U_c$ and $U_s = 5S_s$ (criteria 1 and 2 of the guidance); thus, $S_c = S_s = U_c$. Following eq 4, the Z factor at the LLOQ level defined by the guidance could be derived as $Z = 1 - 3(S_s + S_c)/\text{Abs}(U_s - U_c) = 1 - 3(U_c + U_c)/4U_c = -0.5$. The signal level of the LLOQ (U_s) could be derived for $Z = -0.5$ from eq 4. For ^{14}C plasma AMS analysis, S_s , S_c , and U_c were 0.0909 and 0.0989 and 3.119 dpm/g of C, respectively (Table 4). Therefore, the lowest achievable LLOQ ($Z = -0.5$), U_s , would be 3.50 dpm/g of C or 0.00215 dpm/mL. At this level, $U_s = U_c + 2(S_s + S_c) \approx U_c + 4\sigma_c$, where σ_c is the true standard deviation of blank samples. As demonstrated in Figure 2, U_s at $Z = -0.5$ (blue dashed line) is just above the background signal window $U_c + 3\sigma_c$, but less than $U_c + 3(S_c + S_s)$. This implies that if the bioanalytical guidance is used here, the lowest theoretical LLOQ ($Z = -0.5$) could overlap with the background signal in a large data set.

**Figure 2.** AMS-measured signal of calibration standards of concentration 0 (open squares) and the lowest concentration calibration standard at 0.0159 dpm/mL (open triangles). The dashed line represents the signal at $Z = -0.5$, and the solid line represents the $Z = 0$ level. The dotted lines above and below the symbols represent the $\pm 3\sigma$ levels.

A better and more appropriate, statistically significant criterion for a sample signal clearly distinguishable from the background is $Z \geq 0$.³⁸ We designate the LLOQ derived from eq 4 when $Z = 0$ as $\text{LLOQ}_{Z=0}$ to distinguish it from the experimental LLOQ; the latter is the lowest calibration standard in this study. At $\text{LLOQ}_{Z=0}$, $U_s = U_c + 3(S_s + S_c) \approx U_c + 6\sigma_c$. With better than a 99.999998% probability, a sample signal would be above the background and clearly distinguished in repeated analyses (Table 5). Note that U_c , S_s , and S_c were determined from the analysis of control blanks and lowest calibration standard samples, rather than repeated analysis of a single sample. Thus, as long as the AMS signal maintains the linear relationship with the analyte concentration in the biological matrix, $\text{LLOQ}_{Z=0}$ could be established from analysis of replicates of control blanks and low-concentration calibration standards to extend the range of quantitation. In practice, AMS provides the linear response necessary for this approach.^{30,33,35} The data presented in Figure 1 demonstrate that the AMS response is linear up to 0 dpm/mL. The derived $\text{LLOQ}_{Z=0}$ in this study is 0.00363 dpm/mL for plasma ^{14}C -SCH 900518 analysis on the basis of the experimental S_c , S_s , and U_c calculated using eq 4. As illustrated in Figure 2, the $\text{LLOQ}_{Z=0}$ was better differentiated from the background and was above the lowest achievable LLOQ defined by the guidance if a calibration standard at such a level was available. Thus, using $\text{LLOQ}_{Z=0}$ would alleviate the challenges of preparing calibration standards at a concentration too low to establish the LLOQ for AMS analysis.

If the $\text{LLOQ}_{Z=0}$ was used, the linear range for ^{14}C -SCH 900518 plasma analysis would be extended at the lowest concentration from 0.0159 to 0.00363 dpm/mL. In practice, this means that the reportable plasma ^{14}C -SCH 900518 concentration would be extended from 16 to 48 h for three subjects and from 24 to 48 h for the other three subjects. Furthermore, as shown in Table 6, the mean terminal half-life would be extended from 5.54 to 9.64 h, the mean volume of distribution (V_d) would increase from 304 to 561 L, and the mean clearance (CL) would change from 40.1 to 38.7 L/h. Achieving an LLOQ as low as practical allows for the determination of pharmacokinetic parameters accurately by capturing the terminal phase of elimination of a drug with sufficient data points. In this study assessment of $\text{LLOQ}_{Z=0}$ allowed for an accurate determination of the mean terminal $T_{1/2}$ and the V_d , since data from all six subjects could be

Table 5. LLOQ Derived from Various Z Factor Values and the Corresponding Confidence Level

	Z = -1	Z = -0.5	Z = 0	Z = 0.2	Z = 0.5	exptl (Cal-1)
U_s^a	$U_c + 3\sigma_c$	$U_c + 4\sigma_c$	$U_c + 6\sigma_c$	$U_c + 7.5\sigma_c$	$U_c + 12\sigma_c$	
confidence (%)	99.73	99.9937	99.999998	100	100	
U_s^a (dpm/g of C)	3.40	3.50	3.69	3.83	4.26	4.72
LLOQ (dpm/mL of plasma)	0.00141	0.00215	0.00363	0.00474	0.00806	0.0159

^a U_s values were derived from experimental U_c , S_s , and S_c values (Table 4) using eq 4.

Table 6. PK Parameters Following a Single per os (PO) Dose of 200 mg of SCH 900518 and an iv Dose of 100 μ g (89.7 nCi) of ¹⁴C-SCH 900518 Administered 2 h after the PO Dose

	subject 101	subject 102	subject 103	subject 104	subject 105	subject 106	N	mean
T_{max} (h)	1.92	1.92	3.00	1.00	1.92	3.00	6	2.13
C_{max} (ng/mL)	610	539	302	977	227	523	6	530
AUC_i/D_{po} (h/mL)	10.3	11.2	10.1	15.0	4.48	13.4	6	10.7
LLOQ = 0.0159 (dpm/mL)	half-life (h)	4.23	3.36	7.22	8.62	3.54	6	5.54
V_d (L)	372	226	370	491	204	163	6	304
CL (L/h)	60.9	46.6	35.5	39.5	39.9	18.1	6	40.1
AUC_i [(h μ g)/mL]	1.64	2.15	2.82	2.53	2.51	5.54	6	2.86
AUC_i/D_{iv} (h/mL)	16.4	21.5	28.2	25.3	25.1	55.4	6	28.6
F (%)	62.7	52.1	35.7	59.3	17.9	24.1	6	42.0
LLOQ _{Z=0} = 0.00363 (dpm/mL)	half-life (h)	10.5	10.2	10.3	7.68	13.5	6	9.64
V_d (L)	882	652	515	435	731	149	6	561
CL (L/h)	58.4	44.5	34.6	39.2	37.7	17.8	6	38.7
AUC_i [(h μ g)/mL]	1.71	2.25	2.89	2.55	2.65	5.62	6	2.94
AUC_i/D_{iv} (h/mL)	17.1	22.5	28.9	25.5	26.5	56.2	6	29.9
F (%)	60.1	49.8	34.8	58.9	16.9	23.8	6	40.7

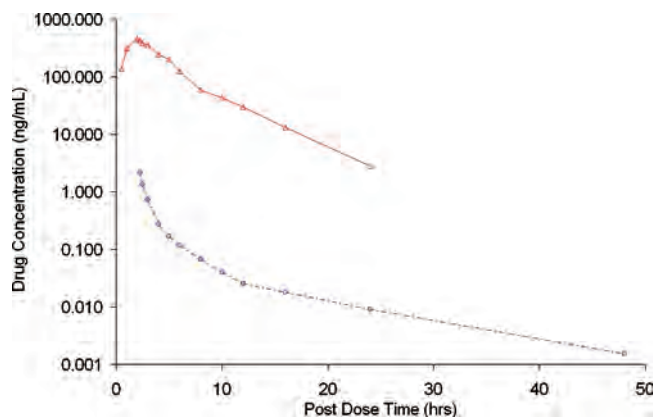


Figure 3. Mean combined SCH 900518 and SCH 782832 plasma concentrations following oral administration of 200 mg of SCH 900518 and an intravenous administration of 100 μ g (89.7 nCi) of ¹⁴C-SCH 900518 2 h later: open triangles, nonradiolabeled drug concentration (oral dose); open circles, ¹⁴C-labeled drug concentration (iv dose).

utilized to the last sampling time point (48 h). Accurate determination of pharmacokinetic parameters of a drug has very important implications to its development program. Evaluation of the Z factor to obtain a statistically significant LLOQ, far below that which could be achieved from the lowest calibration standard, would be extremely helpful to better understand the pharmacokinetics of a drug and its disposition and for modeling and simulation for PK/PD correlation in a large-scale clinical trial

where accurate PK parameters are needed since only limited time point sampling is performed.

Human PK and Absolute Bioavailability Analysis. The total plasma concentrations of ¹⁴C-SCH 900518 and SCH 782832 from the clinical study were analyzed using AMS following the method described in the Experimental Section. The concentration of ¹⁴C-SCH 900518 ranged from 0.00363 to 9.39 dpm/mL (or from 0.00182 to 4.71 ng/mL) from 0 to 48 h post oral dose. Nonradiolabeled SCH 900518 and SCH 782832 plasma concentrations were determined using a validated LC–MS/MS method. The total concentrations ranged from 7.94 to 977 ng/mL following a 200 mg oral dose. The plasma concentrations of SCH 900518 and SCH 782832 were plotted against the corresponding sampling time (Figure 3). The corresponding AUC_i , C_{max} , and T_{max} (Table 6) were obtained following noncompartment model PK analysis using WinNonLin, version 5.2.1 (Pharsight, Cary, NC). The absolute bioavailability ranged from 16.9% to 60.2% with a mean value of 40.7%.

CONCLUSIONS

Absolute bioavailability studies using a ¹⁴C-labeled iv microdose in conjunction with a pharmacologically active oral dose provide a viable, cost-effective, and time-saving means to understand the pharmacokinetic characteristics of a drug in humans. Although validated bioanalytical LC–MS/MS methods have been used in a few cases for plasma concentration determination at microdoses, the use of corresponding validated methods is challenging when the iv microdose is given with a therapeutic oral dose. Therefore, the microdose for AB studies contains a

radiotracer to distinguish it from the oral dose, and AMS is the most suitable technique to measure the ^{14}C concentration. The data from this investigation indicate that methods for AMS analysis of plasma samples following a microdose can be validated using proper calibration standards and regression analysis. The precision (CV) and accuracy (bias) obtained in AMS measurements are similar to those obtained in most LC–MS/MS assays when the nominal concentrations of the standards and QC samples are carefully determined by directly analyzing plasma samples by AMS. Quantitative AMS measurement of drug concentration in a biological matrix does not directly reveal losses that may have occurred during extraction and purification. It is, therefore, necessary to establish a relationship between the true drug concentration and AMS signal. Understanding this relationship permits compensation for experimental variation. As the results reported here demonstrate, highly accurate and precise measurements of plasma ^{14}C -drug concentration can be obtained in microdose studies when a set of calibration standards are included in every sample batch and processed along with the study samples and when the resulting AMS measurements are transformed to drug concentrations using a regression function rather than eq 1. Further statistical analysis of the AMS response of the control and standard samples indicated that a suitable and statistically significant LLOQ can be obtained on the basis of Z factor evaluation at a lower concentration than the LLOQ that could be obtained from the lowest concentration calibration standard. Use of the $\text{LLOQ}_{Z=0}$ for AMS analysis lowered the quantification linear range to a lower concentration than the lowest practical calibration standard and the lowest QC concentration. As a result, the need for preparing an experimentally difficult, very low concentration calibration standard may become superfluous. Use of the Z factor in the present study lowered the LLOQ from 0.0159 to 0.00363 dpm/mL. Thus, reportable plasma concentration data points could be extended from 16 or 24 to 48 h postdose for all six subjects, resulting in a better PK profile overall. In conclusion, analyte-specific ^{14}C -labeled calibration standards, QCs, and clinical samples were quantified by AMS and provided a unified approach for determining recovery at clinically relevant concentrations. The data from this study demonstrate that this approach meets bioanalytical guidance requirements.

■ ASSOCIATED CONTENT

S Supporting Information. Additional information as noted in text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Precision and Accuracy in the Quantitative Analysis of Biological Samples by Accelerator Mass Spectrometry: Application in Microdose Absolute Bioavailability Studies

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Supporting Information

Experimental

Plasma Solvent Extraction for AMS

Extraction of drug-derived material from plasma was accomplished with acetonitrile containing non-radiolabeled SCH 900518, epimer SCH 782832, and metabolites SCH 787443 and SCH 787444 at a concentration of 2 µg/mL each and 0.1% trifluoroacetic acid (v/v) ('extraction solvent'). The non-radiolabeled reference analytes were added to assure specificity during HPLC isolation of the parent drug and epimer as monitored by UV detection. An aliquot of 400 µL standard clinical study plasma sample was transferred into a centrifuge tube pre-filled with 1200 µL plasma extraction solvent. The mixture was vortexed vigorously for approximately 30 seconds and then centrifuged for 30 minutes at 3000g and 4 °C. The supernatant (1500 µL) was transferred into a clean tube. Extraction solvent (1200 µL) was added to the pellet and the pellet was dispersed with a disposable pipette tip. The vortex and centrifuge procedures were repeated as described above. The supernatant (~1200 µL) was removed and combined with the supernatant from the first extraction. The combined supernatant ('plasma extraction supernatant') was evaporated under nitrogen stream with heat controlled at 30 °C to dryness. The dried extracts were stored at –20 °C.

The plasma extraction efficiency covering the drug concentration range in the study samples was determined following the procedure described above but measured prior to drying down the extracted supernatant. Twenty five µL tributyrin solution was added to 80 µL plasma extraction supernatant, before graphitization. The amount of ¹⁴C-SCH 900518 recovered was corrected by subtracting the ¹⁴C level in the control plasma extracts.

Isolation of Analyte Using HPLC

The dried plasma extracts were re-constituted with 250 μ L 7:3 methanol:water (v/v). A 100 μ L reconstituted sample was injected for chromatographic purification of the parent drug and epimer using a Shimadzu Prominence HPLC system (Shimadzu Scientific, Columbia, MD, USA) containing a Shimadzu SPD-10A UV detector and a Shimadzu FRC-10A fraction collector. A binary gradient with constant flow rate of 1.0 mL/min was used for the purification. An Atlantis 100x4.6 mm T3 HPLC column packed with 5 μ m particles (Waters, Milford, MA, USA) and a Zorbax 12.5x4.6 mm SB-C8 guard column packed with 5 μ m particles (Agilent Technologies, Santa Clara, CA USA) provided the separation. The mobile phase consisted of (A) water:trifluoroacetic acid (100/0.01, v/v) and (B) methanol:trifluoroacetic acid (100/0.1, v/v). The initial mobile phase consisted of 68% B, and was maintained at this level for up to 14 minutes. The composition changed from 68% to 80% B from 14 to 14.1 minutes and was maintained at 80% B from 14.1 to 19 minutes. From 19 to 19.1 minutes, the composition jumped to 95% B and was maintained at 95% B to 21 minutes. This was changed back to 68% B at 21.1 minutes and remained at this composition through the remainder of the run (24 minutes) to re-equilibrate the system. The parent drug SCH 900518 and its epimer SCH 782832 co-eluted at approximately 17 minutes. This peak was separated by at least one minute from all other human plasma metabolites. Eight 30-second fractions were collected starting 2.0 minutes before the elution of SCH 900518. Four consecutive fractions corresponding to the center of the SCH 900518 peak as indicated by the UV absorbance monitored at 210 nm were pooled together for AMS analysis.

Sample Analysis by AMS

An aliquot of 150 μ L pooled HPLC fraction from each sample was transferred into separate quartz vials for AMS sample preparation. A known amount of carrier carbon (tributyrin) was added to each quartz vial. The actual amount of tributyrin added to each quartz tube is recorded accurately in the batch file. The actual amount of tributyrin added is used in subsequent calculations. Petroleum-based tributyrin is a non-volatile ^{14}C -depleted liquid and is used to compensate for the depletion of proteins

(hence total C) during extraction of drug-derived material. The graphitization procedure, modeled after Ognibene ¹, converted samples to graphite first by combustion to gaseous CO₂, then reduction to filamentous graphite using Zn and Fe at 500 °C (Figure 1). A 500-kV double stage Pelletron accelerator mass spectrometer equipped with a 40-sample ion source (National Electrostatics Corp, Middleton, WI, USA) was used for the analysis (Figure 2) at Accium BioSciences (Seattle, WA, USA). The major components and operation of AMS instruments have been described in several recent reviews in details ²⁻⁵. In short, negative carbon ions are produced in the cesium sputter ion source and, after low-energy mass analysis, are injected into the tandem accelerator. In the case of ¹⁴C, isobaric interferences are completely eliminated because ¹⁴N does not form stable negative ions. High precision AMS measurements are carried out either by using simultaneous injection or by rapid sequential injection of the isotopes ¹²C, ¹³C and ¹⁴C. Negative ions are attracted to the positive voltage on the terminal at which point they pass through a low pressure gas or a thin carbon foil and are stripped of some of their electrons. Multi-charged positive ions are then further accelerated away from the same positive terminal voltage. The stripping process is also used to destroy molecular interferences, which are the main limitation for conventional mass spectrometry. After the acceleration stage, a magnet selects the most probable charge state. Velocity or energy analyzers provide additional filtering to remove residual background. Finally, identification of the ¹⁴C ions is performed in an ion detector. The isotopic ratio ¹⁴C/¹²C (or ¹⁴C/¹³C) is derived from the ¹⁴C counting rate in the detector and the ¹²C and ¹³C beam currents measured in Faraday cups. A typical AMS sample analysis batch consisted of four certified standards to normalize all measurements, two machine blanks (¹⁴C-free graphite of natural origin) to assess the instrument background, two chemical blanks (samples prepared with ¹⁴C-free substance) to characterize extraneous carbon introduced during sample preparation, if any, and up to 32 clinical study standard, QC, or unknown samples. The measured ¹⁴C/¹²C ratio was expressed as dpm/g C⁵.

Natural ^{14}C Background in Human Plasma and the Effect of Extraction and HPLC Isolation

AMS reports the ^{14}C : ^{12}C isotope ratio in a sample. This ratio can be represented in several common units (1 Modern = 13.56 dpm/g C = 1.176×10^{-12} atom ^{14}C /atom C). All living creatures have a natural ^{14}C abundance that is at equilibrium with the atmosphere and local environment. Contamination from ^{14}C (albeit below the detection limit of scintillation counters) may occur during sample handling, processing and analysis from contact with laboratory surfaces and air. To determine plasma background ^{14}C in humans, plasma samples from 24 healthy male subjects screened in a different clinical study were directly combusted and analyzed by AMS. The natural abundance of ^{14}C in these samples is illustrated in Figure 3. The mean ^{14}C concentration was 0.559 ± 0.005 dpm/mL. This value is consistent with repeated AMS measurement of one blank human plasma sample (0.567 ± 0.018 dpm/mL) reported earlier⁶.

The background radiocarbon was further reduced by protein precipitation and isolation of the SCH 900518 fraction by HPLC. A total of 24 pre-dose human plasma samples collected at four different time points from six subjects were extracted and purified by HPLC as described earlier. The mean concentration of ^{14}C in fractions corresponding to the SCH 900518 region was 0.0000 ± 0.0010 dpm/mL (Figure 3). The standard deviation for the background dropped five fold from 0.005 to 0.0010 dpm/mL compared to direct plasma analysis. This improved the sensitivity and LLOQ of HPLC fractions relative to whole plasma.

Accurate Determination of ^{14}C in Calibration Standards and QC Samples

To validate the method and correctly determine the plasma concentration of study samples, the concentration of the analyte in the calibration standards and QC samples must be accurately determined. In this study, the true concentrations of ^{14}C -SCH 900518 in spiked plasma standards and quality control samples were determined by direct plasma analysis using AMS followed by subtraction of ^{14}C

background in unspiked plasma. These values are more accurate than the nominal concentrations derived from dilutions of the original stock solution for the following reasons. First, the radioactivity of ^{14}C -labeled working standard solutions (usually ~ 100 dpm/mL) are often measured using LSC. The LSC is not an ideal technique at such a “low” range and there is a potential for contamination from high level ^{14}C background that might be present in the facility (0~20 dpm). Second, the compound loss due to nonspecific adsorption could be significant for the ^{14}C -standard at low concentrations. Therefore, reliance on the dilution factor for obtaining radiocarbon concentration could result in erroneous results. In contrast, direct AMS measurement of the final standards and QC samples prepared in plasma involves essentially no sample handling other than routine sample graphitization. As a result, the results are highly reproducible and provide the most reliable and accurate concentrations, as shown in Figure 1 and Table 3 of the article and Figure 4 of the supporting material. The accuracy in direct plasma analysis has been discussed in reference 33 (main article) previously.

Figure Captions

Figure 1. Schematic diagram for reducing CO₂ to graphite.

Figure 2. Schematic layout of a ¹⁴C accelerator mass spectrometer with 500 kV double stage Pelletron accelerators used in this study(*Courtesy of National Electrostatics Corporation*).

Figure 3. ¹⁴C activity of control plasma (open square) and HPLC fraction of pre-dose plasma samples (open triangles). , The dotted lines above and below the symbols represents the $\pm 3\sigma$ level of the signal.

Figure 4. Correlation of ¹⁴C concentration (DPM/mL) of calibration standards (X-axis) with those derived using regression analysis of the AMS measured calibration standards (Y-axis) analyzed together with study samples using the same procedure for sample analysis. Note that the concentration in the calibration standards were determined by directly combusting and analyzing the samples by AMS.

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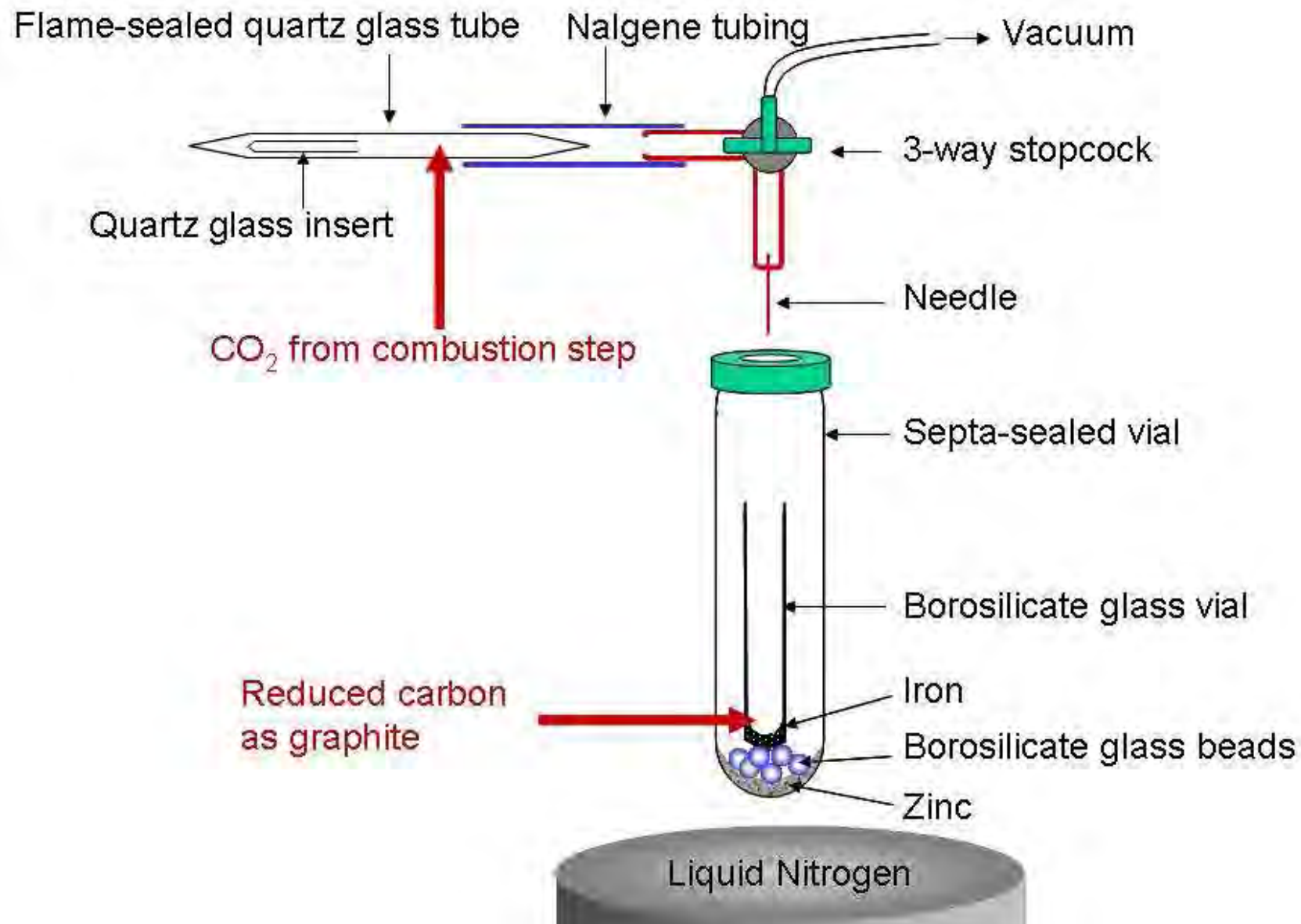


Figure 1. Schematic diagram for reducing CO_2 to graphite.

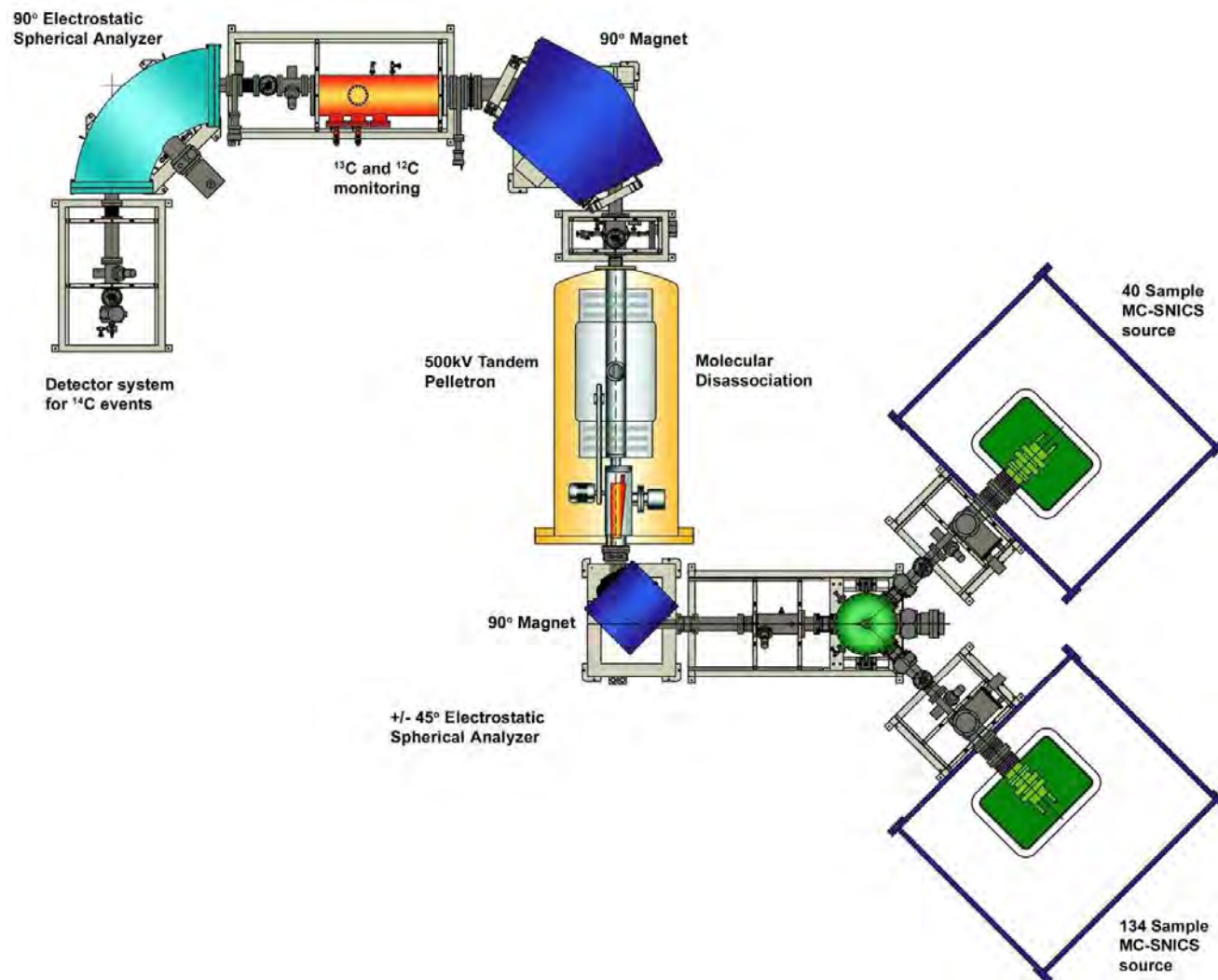


Figure 2. Schematic layout of a ^{14}C accelerator mass spectrometer with 500 kV double stage Pelletron accelerators used in this study (Courtesy of National Electrostatics Corporation).

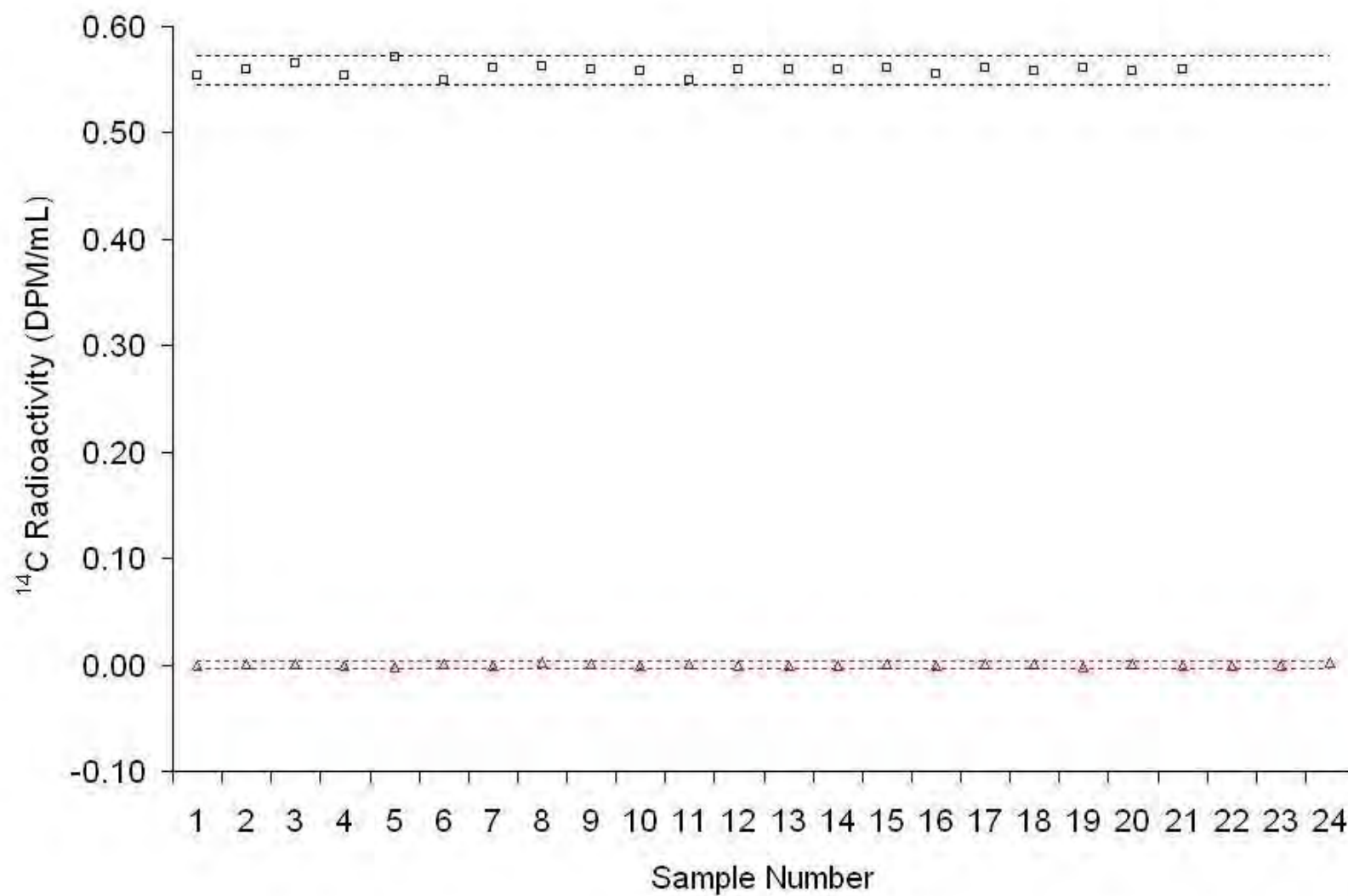


Figure 3. ^{14}C activity of control plasma (open square) and HPLC fraction of pre-dose plasma samples (open triangles). , The dotted lines above and below the symbols represents the $\pm 3\sigma$ level of the signal.

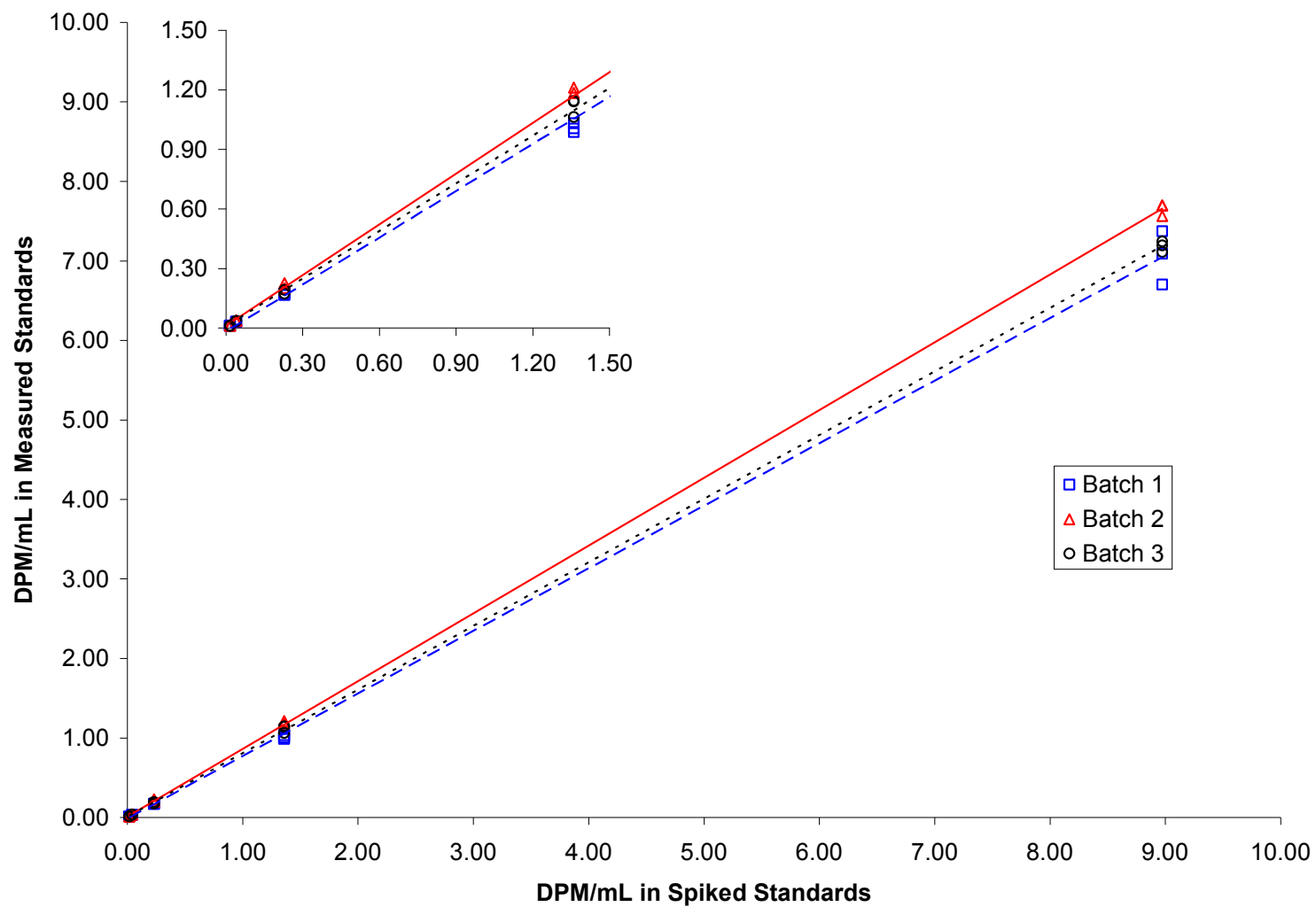


Figure 4. Correlation of ^{14}C concentration (DPM/mL) of calibration standards (X-axis) with those derived using regression analysis of the AMS measured calibration standards (Y-axis) analyzed together with study samples using the same procedure for sample analysis. Note that the concentration in the calibration standards were determined by directly combusting and analyzing the samples by AMS.