



Abstract Metabolites can be both mediators of drug toxicity and efficacy and recent FDA Guidance for Industry strongly recommends that studies leading to *in vivo* drug metabolism in humans be performed as early as feasible. In this light, a complete accounting of significant metabolite(s) in both safety species and humans during clinical testing is a high priority. The quantitative sensitivity of ¹⁴C-Accelerator Mass Spectrometry (AMS) empowers early human metabolism studies to be conducted within Phase I trials with microtrace (nCi) quantities of administered ¹⁴C-labeled drug. Direct atom counting with AMS can reveal the full complement of metabolites with no requirement for internal standards and quantifies dose recovery into excreta (mass balance) with a high degree of reproducibility. These measurements facilitate informed decision-making in and around Phase I studies with incremental added cost to the program using trace radiolabel.

Accelerator Mass Spectrometry is a form of Isotope Ratio MS that traces low abundance carbon-14 for high specificity, but detects them with mass spectrometry for high sensitivity. Atomole levels of ¹⁴C-labeled substrates are traced in milligram-sized samples, enabling metabolism/ADME studies using trace amounts (nCi) of radioactivity in human subjects and pCi quantities in laboratory animals.

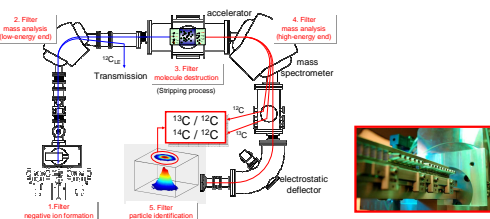


Figure 1. High throughput next generation AMS technology: The BioMICADAS Co-developed by Vitalea Science and the Swiss Federal Institute of Technology. The 200 kV spectrometer produced by the Paul Scherrer Institute and Vitalea Science shown is the next evolution in AMS instrumentation. It delivers superior sensitivity and ruggedness in a laboratory size machine that delivers 24/7 operation through a continuous feed ionization chamber that is isolated from the evacuated spectrometer via a gate valve (photo above on right)

Parameter	¹⁴ C Spectrometer
Specificity	1:10 ¹⁴
Robustness	2.5%
Reproducibility	<1% single sample
Precision	1.5% multiple samples
Stability	<3% over 1 year
LLOQ	0.01 Modern
ULQO	300 Modern
Accuracy/linearity	0.99-1.01
Throughput	20 per hour
Sample Loading	Continuous feed
System Suitability	Once per week

Table 1. Validated Performance of Bio-MICADAS

The validated performance specification are provided in Table 1. The robustness and stability of the spectrometer is evidenced by the requirement for only weekly system suitability tests in GLP operations

Sample Definition and Universal Quantitation

AMS measures an isotope ratio, expressed as ¹⁴C per mass of carbon (¹⁴C/C). A typical Total ¹⁴C measurement requires about 0.4 and 1.0 mg of total carbon (25 µL of plasma). Liquid chromatography fractions are supplemented with carbon carrier to meet this value. AMS instruments receive samples after conversion of an organic specimen to gaseous CO₂ and then reduction to filamentous fullerene (graphite). Although often seen as a barrier, the process is high throughput and exceeding reliable. There is no need for internal reference standards in AMS quantification no matter what the compound, species, matrix, or sample definition as long as all process knowledge is captured and accounted. Accuracy of the ratios is assured by normalization to NIST-traceable external reference standards. Quantitation based upon an intrinsic tag (¹⁴C), matrix independence, and high precision lead to **Universal Precision and Accuracy**. No other analytical method has the sensitivity, specificity and precision of AMS.

The AMS Method: Proven method precision, sensitivity (atomole) and accuracy

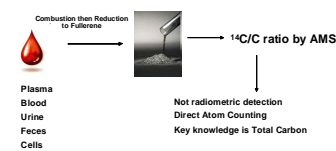


Figure 2. The combustion process means the measurement is independent of matrix. A single operator can prepare 200 graphite targets/day in a GLP system. This stability of the fullerene uncouples the analytical production and definition of metabolic samples from the actual quantitation by the spectrometer, freeing the AMS and chromatography for maximum independent scheduling.

MIST & Metabolite Quantitation

The recent FDA Guidance on Metabolite Safety in Toxicology (MIST) has heightened the need for precise and accurate quantitation of all transformations of compounds in drug development. Quantitation must not only be independent of metabolizing pathways and biological matrices but also independent of animal species. AMS bring the **universal applicability of the isotopic quantitation without extensive specific method development as found in LC-MS measurements, and the assurance that all derivatives of labeled compounds will be quantifiable due to the wide linear range of AMS quantitation**

Example: Quantitative Plasma Metabolite Profiling

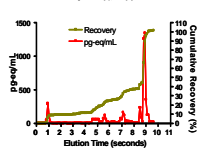
The key to absolute quantitation is to follow **recovery of the ¹⁴C tracer** across the processing method. The quantitative power of AMS is wasted unless all processes are quantitative from the original drawn specimen to the isolated metabolite. The process documentation must note changes in the carbon inventories that can affect isotope ratios. Assay points where the ¹⁴C and carbon inventories must be followed in the preparation of plasma for UPLC-AMS analysis are

- Whole Plasma (100% mark)
- Plasma protein pellet following extraction (extraction recovery/residual)
- Plasma extract following reconstitution in LC solvent (Injectate)
- Summed LC fractions off the LC column (column recovery)

The 100% mark is established through analysis of whole plasma. The plasma is then processed for UPLC separation: typically plasma proteins are precipitated with two additional equivalents of acetonitrile and proteins separated by vortexing. Unlabeled parent compound and/or specific metabolites can be added during the protein precipitation or resuspension to act as a UV markers.

The supernatant is removed and taken to dryness under reduced pressure. The dried solids are reconstituted in the appropriate solvent and injected on a UPLC systems with variable time fraction collector with minimal 6-ε resolution. A second aliquot is placed into a sample combustion vial for separate direct measurement of ¹⁴C content in the injectate to quantify summed recovery off the column.

Plasma Metabolite Profile: 2.5-24 hr Pool



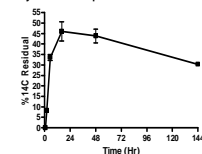
UPLC Quantitative Summation - An example is shown in the below figure, where the ¹⁴C content of a 20 µl sample of the injectate is compared to the separated fractions and the **sum of the fractions of a UPLC elution**. Quantitative summation assures the analyst that no losses or contaminations occurred during chemical species definition.

Table 2 traces the recovery across the process for a human plasma sample. The UPLC injectate had a ¹⁴C concentration that was 91% that of the unprocessed plasma. Again, variable width fractions were quantified by AMS because few early elutes were less important. The column recovery adds up to 100%. In this instance, one would correct the quantified metabolite profiles by a factor of 1.10 to account for the 9.4% loss in processing the plasma prior to LC separation.

	[C] (MG/ML)	R ₁ (MODERN)	R ₂ (MODERN)	LLOQ (PM °C)	TRACER (PM °C)	FRACTION (%)
PLASMA	43	2.91±0.42	1.061±0.019	0.8	7.8	100
PELLET	37	1.25	1.061±0.019	0.7	0	4.2
INJECTATE	6	3.73	0.096±0.008	0.12	7.1	90.6
COLUMN			0.096±0.008	0.12	7.0±0.1	98.5±1.5

Time dependency in extraction of plasma

Assay 312 Time Dependence of Plasma Extraction



A subsample of the **plasma protein pellet** post precipitation is taken by mincing the firm pellet with a razor blade or similar device. A 1-5 mg piece of the pellet is dried under vacuum and analyzed for ¹⁴C contents above natural background.

UPLC as a component of Universal Quantitation

Reproducibility - UPLC Stability

UPLC separation is well suited to high throughput AMS because the separations are complete within 10 minutes and the fractions' solvent volumes are much smaller than from HPLC flow rates. The solvents are evaporated just to dryness in a clean vacuum centrifuge, leaving at most tens of micrograms of carbon in the residue. About 1 mg of carbon carrier compound is added prior to combustion and reduction.

A pair of cross-validated Waters AcuityTM UPLC instruments are used at Vitalea Science for metabolic analyses and have very high reproducibility. Figure 3 shows AMS quantitation for a single metabolite peak from urine separated on different days. AMS provides an uncertainty estimate for each measurement based on the number of recorded ¹⁴C atoms (CV = 1/N) and these uncertainties are shown for each datum in the figure. There should be two of the six data replicates that do not overlap at the 1 sigma uncertainties, but there is only one that one occurs at the sharply rising start of the peak. Thus, these UPLC's are reproducible with AMS quantitation to "better than statistical" accuracy.

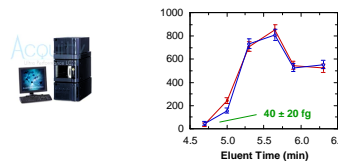
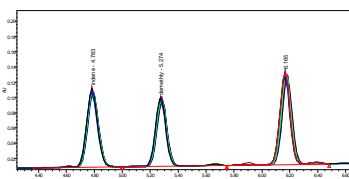


Figure 3. A test of UPLC-AMS reproducibility shows a metabolite peak in urine samples separated by UPLC and measured by AMS on different days. Error bars represent the AMS counting uncertainty at 1 sigma and should overlap only at 4 of the 6 points. The only non-overlap occurs at the rising edge of the peak.

An overlay of 11 chromatograms run in a 6 hr session is shown in Figure 4. The stability ensures the integrity as specific fraction collections and comparability of interspecies comparisons or metabolite profiling data collected over time.



Interspecies Profiling

Universal Quantitation means that robust and absolute abundance are obtained regardless of changes in species matrix. This means minimal method development other than determination of analyte recovery.

Metabolites play roles in distribution and clearance, affect the pharmacokinetics of the parent compound and are potential pharmacological or toxicological agents in themselves. Quantitation of a metabolite is important if the metabolic transformation greatly affects the bioavailability and/or the bioequivalence of the compound for the desired pharmacological effect. The relative amount of a compound's metabolism also affects toxicity either positively or negatively. Preclinical studies reveal mammalian metabolic pathways and may identify potentially worrisome endpoints, while human cell or tissue cultures indicate whether the preclinic is predictive of the clinic. Nonetheless, determination of the complete set of circulating human metabolites of a drug candidate as early in development as possible has importance to the pharmacology of the compound as well as its future suitability as a pharmaceutical. While quantitative clinical metabolite analyses are often accomplished during clinical ADME studies, once sufficient radiolabeled compound is available, human metabolite profiles for comparison to safety species can greatly affect the direction and speed of clinical development.

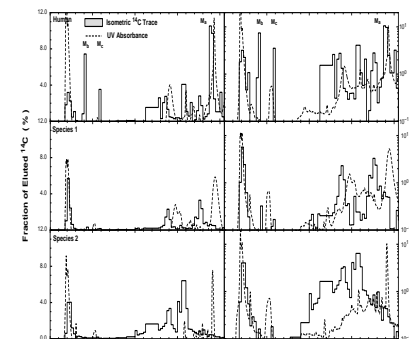


Figure 4. UPLC-AMS traces of human (top) and two animal species plasmas after dosing with a ¹⁴C-labeled compound are shown as fraction of the total eluted ¹⁴C signal (shaded skyline) and for UV absorbance (dashed line). The left frames have a linear ¹⁴C scale which is given in log scale to the right. A metabolite marker is seen in the UV trace

Early Stage ADME - Barriers Dropping

One obstacle to obtaining early ADME readouts in Phase 1 stems directly from concerns associated with added costs for a compound that may well fail at later stages. Vitalea Science believes the advantages of early human information, enabled by the low radiologic exposures of AMS deliver value that far exceeds the monetary considerations. ADME readouts in Phase 1 provide insights that cannot be obtained with standard bioanalytical analyses - insights that could salvage the project through a greater understanding of metabolism and disposition issues not predicted by *in vitro* and animal studies as well as insights that could be applied to backup compounds in the same class. There is **no substitute for human ADME**.

For early Phase ADME, Vitalea advocates a **bioanalytical approach that employs flexible bioanalysis protocols designed to minimize analytical costs through hybridization of analytical platforms (AMS and traditional radioanalysis) and a staged approach to the number of type of analyses.**

The BioMICADAS AMS is the wave of the future in lower cost radioanalysis. For more information contact

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Correlation of AMS and LC/MS specific molecular analysis

While AMS is seen as an ADME tool, it can also be the only detector of choice in many studies. Implicit within this application is the knowledge that UPLC AMS and LC/MS, when operating in concentrations ranges accessible to AMS, will provide similar results. Below in Figure AAA are the results of a comparison of AMS and LC/MS results for a dose that was labeled at less than one ¹⁴C molecule per three hundred (1:300). Even though AMS detects only the ¹⁴C labeled molecules, very concordant data were obtained between the two instruments. The AMS bars (black and red) represent duplicate samples individually speciated by UPLC and reduced to graphite. The %CV for the duplicate AMS injections was <2%, which far exceeded normal analytical expectations for precision.

