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Methods and Applications of HPLC-AMS (WBio 5)

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Pharmacokinetics of physiologic doses of nutrients, pesticides, and herbicides can easily be traced in humans using a ^{14}C-labelled compound. Basic kinetics can be monitored in blood or urine by measuring the elevation in the ^{14}C content above the control predose tissue and converting to equivalents of the parent compound. High Performance Liquid Chromatography (HPLC) is an excellent method for the chemical separation of complex mixtures whose profiles afford estimation of biochemical pathways of metabolism. Compounds elute from the HPLC systems with characteristic retention times and can be collected in fractions that can then be graphitized for AMS measurement. Unknowns are identified by coelution with known standards and chemical tests that reveal functional groupings. Metabolites are quantified with the ^{14}C signal. Thoroughly accounting for the carbon inventory in the LC solvents, ion-pairing agents, samples, and carriers adds some complexity to the analysis. In most cases the total carbon inventory is dominated by carrier. Baseline background and stability need to be carefully monitored. Limits of quantitation near 10 amol of ^{14}C per HPLC fraction are typically achieved. Baselines are maintained by limiting injected ^{14}C activity <0.17 Bq (4.5 pCi) on the HPLC column.

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1. Introduction

Using AMS to analyze HPLC eluent exploits the strengths of each analysis method while minimizing the weaknesses. Complex chemical mixtures such as metabolites of pesticides, vitamins or pharmaceuticals can be separated and the components identified with HPLC. Separation is accomplished through appropriate selection of mobile phase conditions (solvents, flowrates, elution gradients) and chromatographic (stationary phase) column (resin bonding, support choices, bore diameter, length). Identification is accomplished by co-elution with known standards. Obviously, an unexpected metabolite complicates identification since a purified standard is often unavailable. Traditional HPLC quantitation uses UV/Vis absorbance of a compound or radioactivity detection in a flow-through detector or liquid scintillation counter (LSC). Not all compounds possess useful chromophores to aid in detection by UV/Vis and many non-metabolites do absorb at commonly used wavelenghts in typical biological samples such as plasma or urine. Flow through scintillation detectors require high levels of activity in the parent compound because the volumes of biological samples loaded on the column are limited. Chromatography eluent can be collected as separate fractions in a time series (often 30 or 60 second increments) and measured by LSC. This approach generally has better sensitivity than a flow through detector, but is still inadequate for studying relevant exposures to reasonably labelled compounds.

The strength of AMS is quantitation of small amounts of radiolabeled metabolites. Using a radiolabeled test compound insures specificity of metabolite origin. The metabolites contain the label and are distinct from all other chemical species in the sample. In the case of ^{14}C, the label can usually be incorporated into a stable position in the test compound. AMS measures a perturbation of ^{14}C label on a 10 amol background (carrier carbon per

AMS sample) while decay counting measures perturbations above a 10-30 DPM background, a difference of 4 orders of magnitude in sensitivity. Since AMS measures isotope ratios it cannot provide any information on chemical structure or identity, it only detects differences in isotope enrichment. The strength of HPLC-AMS is compound separation and identification with the chromatography system and superior quantitation of small or low specific activity compounds with AMS.

2. Chemical Separation and Identification

All chromatography procedures aim to separate compounds of interest sufficiently well to achieve identification while minimizing analysis times. In practice, these often competing concerns are balanced to achieve a reasonable compromise. Initially one must make decisions about which metabolites are most important and choose appropriate separation conditions such as flow rate, column type and solvents. Although this assumes *a priori* knowledge of potential metabolites, a working hypothesis can guide the initial work. However, an unknown or unexpected metabolite has been found in almost every case we encountered. Chromatography conditions are then determined using pure standards without radiolablels. Conventional detection systems such as UV/Vis absorbance are used to monitor the elution of the individual standards and mixtures, even in the presence of co-eluting labeled analytes. The quantity of standard must be sufficient to produce a clear UV absorbance peak but still small so that contributions to the carbon inventory are negligible. Experience indicates ^{14}C peaks are wider than the UV absorbance peaks (several minutes vs. less than a minute) due to the coarse elution fraction intervals, greater dynamic range and lower background of AMS.

3. AMS Concerns

3.1 Carbon inventory

The ability to accurately quantify the level of ^{14}C-labeled tracer in an HPLC elution fraction requires knowledge of the ^{14}C content and the mass of each component of the fraction (injected sample, HPLC standards, solvent residue, ion-pairing agents, carbon carrier). We want situation where

$$R_{sample} = \frac{^{14}C_{tracer} + {}^{14}C_{tissue} + {}^{14}C_{carrier} + {}^{14}C_{solvent\ residue} + {}^{14}C_{unknown}}{C_{tracer} + C_{tissue} + C_{carrier} + C_{solvent\ residue} + C_{unknown}} \quad (1)$$

reduces to

$$R_{sample} = \frac{^{14}C_{tracer} + {}^{14}C_{carrier}}{C_{carrier}} \quad (2)$$

by judicious selection of chromatography conditions and sample preparation. The sample carbon mass is limited by the concentration and volume of the injected solution. Typical injection volumes are 5-200 µL with 1-5% carbon content (≤1 mg) for column diameters 2-5 mm. Removal of proteins or other matrix components by heat or solvent induced precipitation can be done before injection to reduce carbon mass and prevent clogging the column if the tracer is kept in solution. The sample (tracer + tissue) should have the majority of the ^{14}C but negligible mass. The HPLC standards should have contemporary or lower carbon ratios but masses too low to affect predose backgrounds. Solvent residue can usually be dealt with by avoiding carbon containing salts or non-volatile organic solvents. If an ion-pairing agent is needed, consider using a volatile one (e.g., trifluoroactic acid) that is removed during sample drying or an agent with a low ^{14}C ratio that can serve as a carbon carrier (e.g., 1-mL of 5mM tetrabutylammonium dihyrdrogen

phosphate contains 0.96 mg C). The carbon carrier mass should dominate and possess a low ^{14}C ratio (we use 10 pMc tributyrin).

3.2 Background, controls, and LOQ

Working with the AMS levels of ^{14}C requires use of a HPLC dedicated to AMS separations only. It is best to buy a new HPLC at the start of AMS work and replace the chromatogrphy column with each new set of experiments. Chromatogrphy columns are inexpensive compared to the costs of AMS sample preparation, measurement, and analysis. No hot material is allowed in the system and all samples must be checked for activity before injection on the column. A practical working limit is <10 DPM (0.17 Bq, 4.5 pCi) ^{14}C on the column at any time. This high level would only be used for a large bore column (>5-mm; semi-preparative) and a complex mixture where the label is distributed across many metabolites. A 2-mm bore column and relatively simple mixture of metabolites can be limited to 1-2 DPM. The ratios of all solvents, buffer salts, and ion-pairing agents must be checked prior to use on the HPLC to avoid inadvertant contamination.

Check the system background by pooling elution fractions of solvents alone in groups of 5-10 min and measuring the isotope ratios. After checking the background of the HPLC system, control samples (typically predose samples of the tissues from the dose subjects) are analyzed. The cold standards are coinjected with the control sample and a full elution sequence is collected, converted to graphite targets [1], and measured. This histogram spectrum is the baseline from which ^{14}C elevation is measured. If the baseline is flat, an average of the ^{14}C level in the fractions can be used as the background. If an elution gradient is used to separate metabolites on the LC, the solvent residue needs to be checked

very carefully to ensure the carbon inventory in the fractions does not vary over the course of the elution. A probablility plot of the predose control fractions is useful for determining the median, mean, and distribution of the background samples. The limit of quantitation (LOQ) for the HPLC-AMS fractions is determined by the average of the baseline fractions plus two standard deviations, typically in the vicinity of 2-20 amol ^{14}C (Fig.1).

4. Working with Real Samples

4.1 Dermal exposure to ^{14}C atrazine

Atrazine is among the most commonly used herbicides world wide. Application of ring-labeled ^{14}C atarzine was designed to simulate occupational exposure to farm workers for determination of metabolic biomarkers. A conventional assay could then be designed to detect the dominant metabolite class and monitor field exposures to workers. Subjects received a dermal dose of either 0.167 mg (6.45μCi) or 1.98 mg (24.7 μCi) and urine was collected for a week and measured for metabolite determination [2]. The activity of urine was too low to measure separated fractions by the planned liquid scintillation technique. AMS easily measured the quantity of ^{14}C in HPLC fractions, in fact many urine samples from the high dose group had levels higher than prefered for AMS analysis. Figure 2 depicts the atrazine metabolite profile in urine from a subject in the low dose group. The UV absorbance spectrum includes peaks from four standards at 19.3, 23.5, 27.3 and 39.1 min, didealkylatrazine mercapturate, deisopropylatrazine mercapturate, deisopropylatrazine mercapturate, and atrazine mercapturate, respectively. The UV peaks do not always correspond to ^{14}C peaks and these artifacts are unrelated to the atrazine metabolites. The strong ^{14}C signal at 39 min was identified as atrazine mercapturate while the signal at 11 min did not have a corresponding standard. In most cases the AMS histogram peaks are wider than the absorbance peaks. Atrazine is a difficult compound to monitor because it

has so many metabolites, many of which are chemically similar and elute near each other. Based on the metabolite profiles measured in this study it appears that the mercapturate class of metabolites are a suitable biomarker for dermal atrazine exposure.

4.2 Tracing vitamin metabolism with ^{14}C beta carotene

Beta carotene is precursor to vitamin A and a popular dietary supplement. We intrinsicly labeled spinach using $^{14}CO_2$ and a growth chamber. The spinach was harvested and dietary nutrients were removed from the leaves for use in human metabolism studies. The specific activity of the beta carotene used in this study was 0.35 mCi/mmol (0.6% of the molecules if single label)[3]. The beta carotene was administered orally as a 200 nCi (300 μg) dose and blood was collected over a period of several months. Plasma was separated from the erythrocytes and measured to determine general kinetics. Plasma proteins were precipitated using ethanol and a complex array of metabolites were extracted using acid/base conditions with a hexane upper phase. Separation of metabolites into chemical classes by acid/base extraction conditions simplified the metabolite mixtures so that only 3 or 4 metabolites appeared in the elution fractions. After doing several analyses of the full elution series we were able to switch to collecting only the peaks, significantly reducing the AMS sample prep and analysis. This work was done with a new HPLC using a narrow-bore column (3.0 mm) and lower flow rate (0.45 mL/min) than the atrazine study described earlier. The reduction in background carbon and use of a new system pushed the limit of quantitation to the 1-2 amol range (Fig. 1), an order of magnitude below the atrazine study.

5. Conclusions

HPLC-AMS takes advantage of the strengths of each analysis method: Metabolite separation and identification with HPLC and quantitation with AMS. This principle can be applied to any chromatographic separation system by limiting the the isotope content to AMS ranges and understanding the carbon inventory of the final AMS targets. Pharmacokinetics of physiologic doses can be traced and metabolites measured accurately by utilizing the sensitivity of AMS. Graphite conversion of sample fractions is a cumbersome step in the current method. An improved ion source which could accept gas injection of a pyrolized HPLC or GC output would be a dramatic improvement and propel the technique into the mainstream [4].

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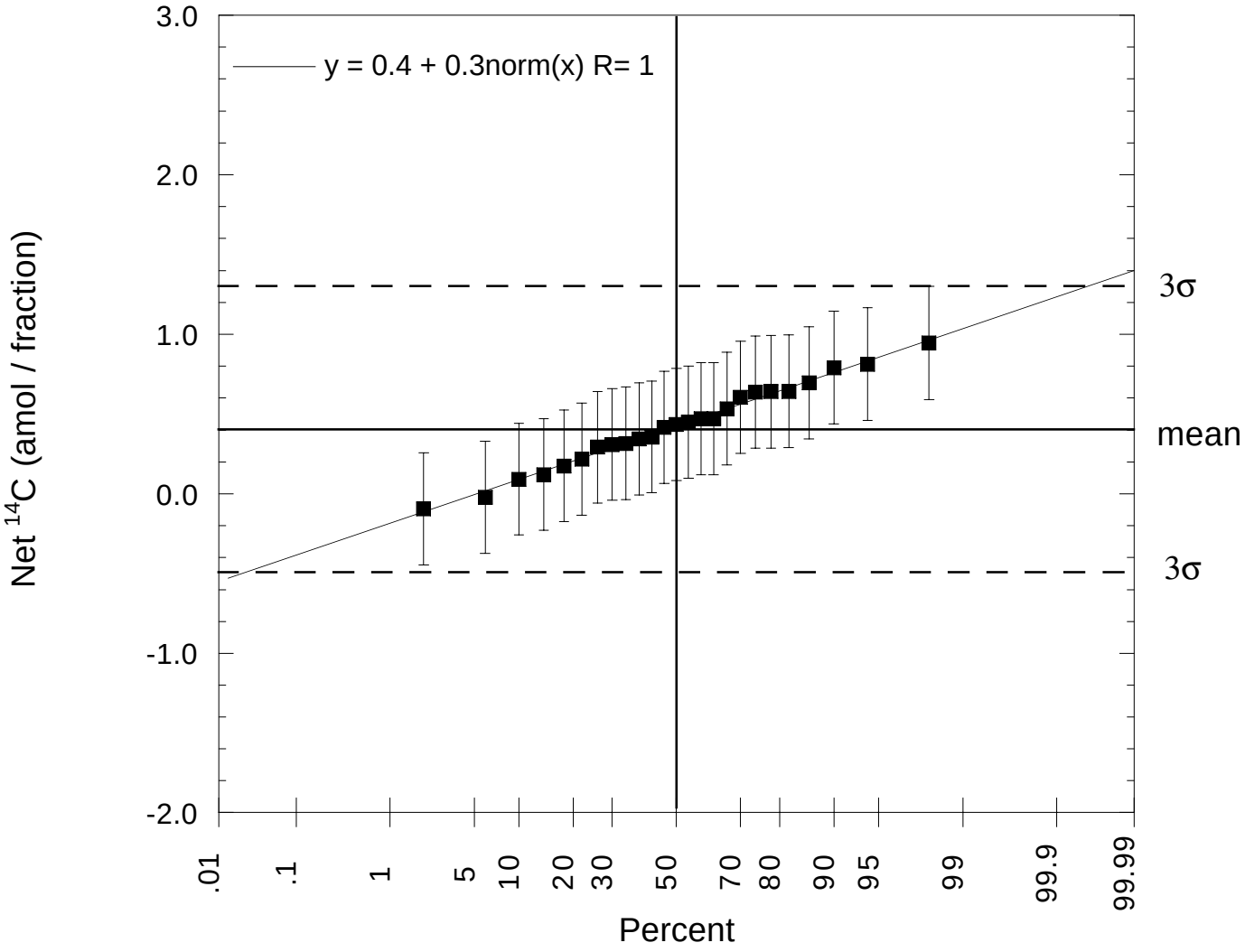
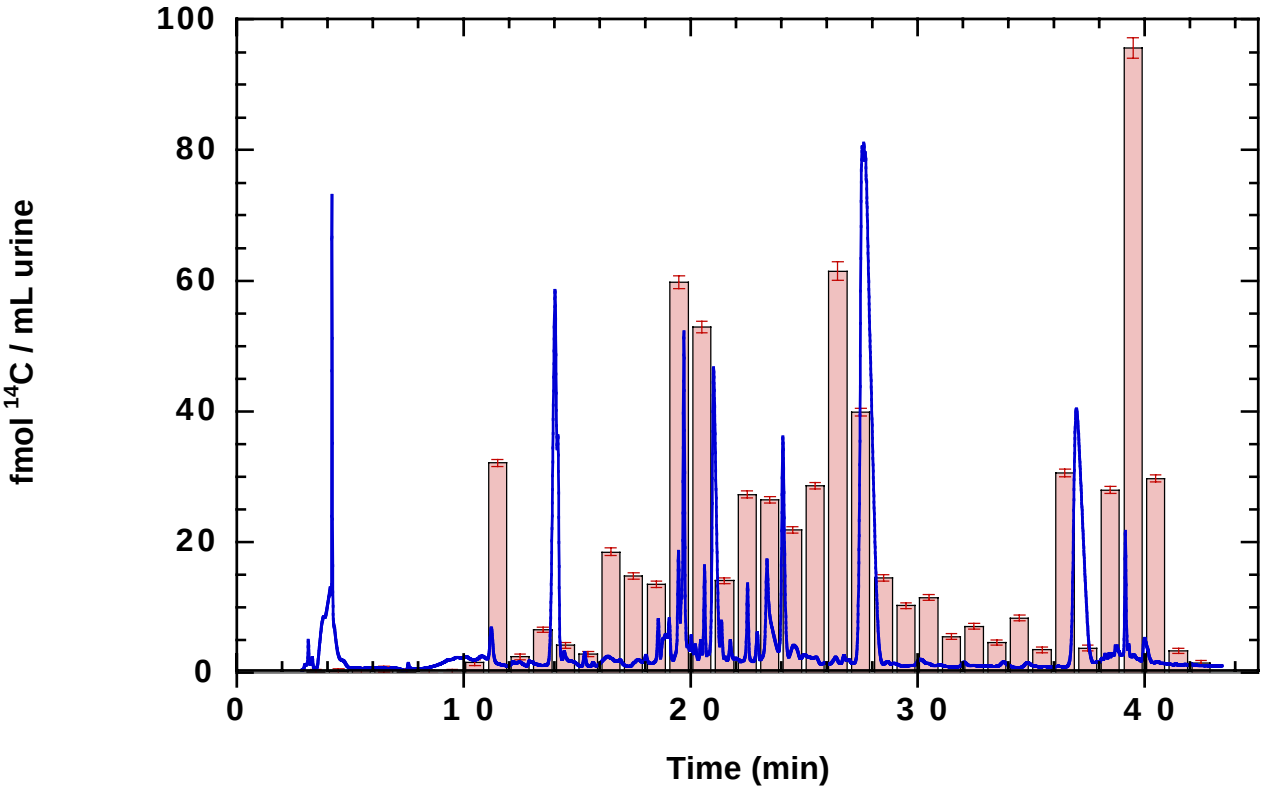
Fig. 1 Predose HPLC fractions of acid and neutral fractions pooled (n=25) 0.4± 0.3 amol of ^{14}C. The slope of the probablility plot corresponds to the standard deviation and a straight line indicates a Gaussian distribution. This unusually low background was achieved using a new HPLC system. A reliable limit of quantitation (LOQ) is 1-2 amol of ^{14}C per fraction above the carrier.

Fig. 2. UV absorbance spectrum superimposed on AMS ^{14}C histogram of 4-8 h urine sample from 24 h dermal exposure to atrazine (0.167 mg, 6.45μCi).


y = 0.4 + 0.3norm(x) R= 1
3.0
2.0
1.0
0.0
-1.0
-2.0
3σ
mean
3σ
Net ^{14}C (amol / fraction)
.01
.1
1
5
10
20
30
50
70
80
90
95
99
99.9
99.99
Percent


100
80
60
40
20
0
0
10
20
30
40
fmol 14C / mL urine
Time (min)