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## Methods and applications of HPLC–AMS

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### Abstract

Pharmacokinetics of physiologic doses of nutrients, pesticides, and herbicides can easily be traced in humans using a <sup>14</sup>C-labeled compound. Basic kinetics can be monitored in blood or urine by measuring the elevation in the <sup>14</sup>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 tentatively identified by co-elution with known standards and chemical tests that reveal functional groupings. Metabolites are quantified with the <sup>14</sup>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 <sup>14</sup>C per HPLC fraction are typically achieved. Baselines are maintained by limiting injected <sup>14</sup>C activity <0.17 Bq (4.5 pCi) on the HPLC column. © 2000 Elsevier Science B.V. All rights reserved.

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### 1. Introduction

Using AMS to analyze high performance liquid chromatography (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, flow rates, elution gradients) and chromatographic (stationary phase) column (resin bonding, support choices, bore diameter, length). Identification is accomplished by co-elution with known standards. Traditional HPLC quantitation uses UV/Visible absorption of a compound or radioactivity detection in a flow-through

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detector or liquid scintillation counter (LSC). Not all compounds possess useful chromophores to aid in detection by UV/Visible absorption and many non-metabolites do absorb at commonly used wavelengths 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 s increments) and measured by LSC. This approach generally has better sensitivity than a flow through detector, but is usually still inadequate for studying environmental exposures to reasonably labeled compounds ( $^{14}\text{C}$  specific activity in the range 10–200 mCi/mmol).

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  $^{14}\text{C}$ , the label can usually be incorporated into a stable position in the test compound. AMS measures a perturbation of  $^{14}\text{C}$  label on a 10 amol background (carrier carbon per AMS sample) while decay counting measures perturbations above a 10–30 DPM (70–210 fmol  $^{14}\text{C}$ ) background, a difference of four 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 determined using pure standards without radiolabels and conventional detection systems such as UV/Visible absorption are used to monitor the elution of the individual standards and mixtures. Mixtures of these pure standards are generally co-injected with the samples to monitor the elution of known peaks. The quantity of standard must be sufficient to produce a clear UV absorption peak but still small so that contributions to the carbon inventory are negligible. Experience indicates  $^{14}\text{C}$  peaks are wider than the UV absorption peaks (several minutes versus less than a minute) due to the coarse elution fraction intervals, greater dynamic range and lower background of AMS.

## 3. AMS concerns

### 3.1. Background, controls and LOQ

Working with AMS levels of  $^{14}\text{C}$  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 chromatography column with each new set of experiments. Chromatography 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)  $^{14}\text{C}$  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 among many metabolites. A 2-mm bore column and relatively simple mixture of metabolites can be limited to 1–2 DPM. The  $^{14}\text{C}$  content of all solvents, buffer salts, and ion-pairing agents must be checked prior to use

on the HPLC to avoid inadvertent 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 co-injected 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  $^{14}\text{C}$  elevation is measured. If the baseline is flat, an average of the  $^{14}\text{C}$  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 probability 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  $^{14}\text{C}$  (Fig. 1).

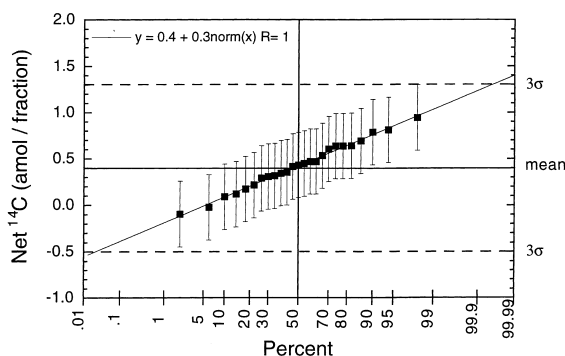


Fig. 1. Predose HPLC fractions of acid and neutral fractions pooled ( $n = 25$ )  $0.4 \pm 0.3$  amol of  $^{14}\text{C}$ . The slope of the probability plot corresponds to the standard deviation of a normal distribution ( $0.3 \text{ norm}(x)$ ) and a straight line indicates a Gaussian distribution. A reliable limit of quantitation (LOQ) is 1–2 amol of  $^{14}\text{C}$  per fraction above the carrier, based on the mean of the distribution plus three standard deviations ( $0.4 + 0.9 = 1.3$  amol  $^{14}\text{C}$ ). This unusually low background was achieved using a new HPLC system.

### 3.2. Carbon inventory

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

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

reduces to

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

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  $\mu\text{l}$  with 1–5% carbon content by mass ( $\leq 1$  mg) for column diameters 2–5 mm. Urine ( $\sim 1$  wt% C) and plasma (4.2 wt% C) are typical tissues suitable for HPLC with little or no processing. 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  $^{14}\text{C}$  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., trifluoroacetic acid) that is removed during sample drying or an agent with a low  $^{14}\text{C}$  ratio that can serve as a carbon carrier (e.g., 1-ml of 5 mM tetrabutylammonium dihydrogen phosphate contains 0.96 mg C). The carbon carrier mass should dominate and possess a low  $^{14}\text{C}$  ratio (we use tributyrin containing 9 amol  $^{14}\text{C}/\text{mg C}$ ). Every project is

different and it is productive to work with an experienced HPLC separation chemist when designing a specific HPLC–AMS separation–quantification procedure.

#### 4. Working with real samples

##### 4.1. Dermal exposure to $^{14}\text{C}$ atrazine

Atrazine is among the most commonly used herbicides world wide. Clinical application of ring-labeled  $^{14}\text{C}$  atrazine 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 atrazine (6.45  $\mu\text{Ci}$ ) or 1.98 mg atrazine (24.7  $\mu\text{Ci}$ ) 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  $^{14}\text{C}$  in HPLC fractions, in fact many urine samples from the high dose group had levels higher than preferred for AMS analysis. Fig. 2 depicts the atrazine metabolite

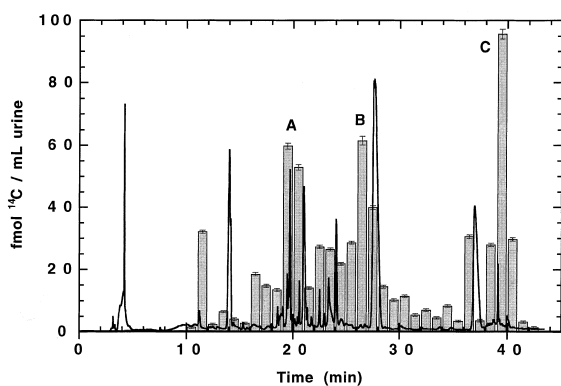


Fig. 2. UV absorption spectrum superimposed on AMS  $^{14}\text{C}$  histogram of 4–8 h urine sample from 24 h dermal exposure to atrazine (0.167 mg, 6.45  $\mu\text{Ci}$ ). Potential biomarkers for dermal atrazine exposure were tentatively identified as didealkylatrazine mercapturate (A), deethylatrazine mercapturate (B), and atrazine mercapturate (C).

profile in urine from a subject in the low dose group. The UV absorption spectrum includes peaks from four standards at 19.3, 23.5, 27.3 and 39.1 min, didealkylatrazine mercapturate, deisopropylatrazine mercapturate, deethylatrazine mercapturate, and atrazine mercapturate, respectively. The UV peaks do not always correspond to  $^{14}\text{C}$  peaks and these artifacts are unrelated to the atrazine metabolites. The strong  $^{14}\text{C}$  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 is a suitable biomarker for dermal atrazine exposure.

##### 4.2. Tracing vitamin metabolism with $^{14}\text{C}$ beta carotene

Beta carotene is a precursor to vitamin A and a popular dietary supplement. We intrinsically labeled spinach using  $^{14}\text{CO}_2$  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  $\mu\text{g}$ ) 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 isotope content to AMS ranges and understanding the carbon inventory of the final AMS samples. 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 pyrolyzed HPLC or GC output would be a dramatic improvement and propel the technique into the mainstream [4,5].

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